



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US90/03141 <b>(22) International Filing Date:</b> 4 June 1990 (04.06.90)  <b>(30) Priority data:</b> 361,912                      6 June 1989 (06.06.89)                      US  <b>(71) Applicant:</b> CALIFORNIA BIOTECHNOLOGY INC. [US/US]; 2450 Bayshore Parkway, Mountain View, CA 94043 (US).  <b>(72) Inventors:</b> SCHILLING, James, W., Jr. ; 247 Byron Street, Palo Alto, CA 94301 (US). PONTE, Phyllis, A. ; 1418 Wildrose Way, Mountain View, CA 94043 (US). COR- DELL, Barbara ; 4051 Ben Lomond Drive, Palo Alto, CA 94306 (US).		<b>(74) Agents:</b> BOZICEVIC, Karl et al.; Irell & Manella, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).  <b>(81) Designated States:</b> AT (European patent), BE (European patent), CA, CH (European patent), DE (European pa- tent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (Euro- pean patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> RECOMBINANT ALZHEIMER'S AMYLOID PROTEIN  <b>(57) Abstract</b>  DNA sequences encoding $\beta$ -amyloid-related proteins associated with Alzheimer's disease are disclosed. Also provided herein is a DNA sequence encoding a novel protease inhibitor. These sequences are used in producing or constructing recombinant $\beta$ -amyloid core protein, $\beta$ -amyloid-related proteins and recombinant or synthetic immunogenic peptides. Antibodies generated against the recombinant proteins or immunogenic peptides derived therefrom can be used for cerebral fluid or serum protein diagnosis of Alzheimer's disease.		

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RECOMBINANT ALZHEIMER'S AMYLOID PROTEIN

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Technical Field

The invention generally relates to the diagnosis and treatment of Alzheimer's disease. More specifically, it relates to the characterization and use of materials related to amyloid protein deposits associated with Alzheimer's disease, and to a specific DNA sequence encoding a novel protease inhibitor.

Background Art

The demography of Alzheimer's disease is becoming progressively better understood. It is estimated that over 5% of the U.S. population over 65 and over 15% of the U.S. population over 85 are beset with this disease (Cross, A.J., Eur J Pharmacol (1982) 82:77-80; Terry, R.D., et al, Ann Neurol (1983) 14:497-506). It is believed that the principal cause for confinement of the elderly in long term care facilities

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is due to this disease, and approximately 65% of those dying in skilled nursing facilities suffer from it.

To confound the problem that therapy is at present a matter of experimentation, diagnosis is also  
5 unreliable. There is no straightforward diagnostic test, and diagnosis is made by a series of evaluations based on negative results for alternative explanations for the symptomologies exhibited. Assuming that the presence of the disease can be assessed accurately after  
10 death by autopsies of the brain, current results show that present diagnostic methods among living individuals carry an approximately 20% rate of false positives.

It would be extremely helpful in effecting appropriate care for patients and in developing  
15 therapies to have a straightforward assay method for diagnosing the presence of Alzheimer's disease. The invention described below provides an approach to this diagnosis.

Certain facts about the biochemical and  
20 metabolic phenomena associated with the presence of Alzheimer's disease are known. Two morphological and histopathological changes noted in Alzheimer's disease brains are neurofibrillary tangles (NFT) and amyloid deposits. Intraneuronal neurofibrillary tangles are  
25 present in other degenerative diseases as well, but the presence of amyloid deposits both in the interneuronal spaces (neuritic plaques) and in the surrounding microvasculature (vascular plaques) seems to be characteristic of Alzheimer's. Of these, the neuritic  
30 plaques seem to be the most prevalent (Price, D.L., et al, Drug Development Research (1985) 5:59-68). Plaques are also seen in the brains of aged Down's Syndrome patients who develop Alzheimer's disease.

The protein which makes up the bulk of these  
35 plaques has been partially purified and sequenced.

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Plaque-rich brains of deceased Alzheimer's patients have been used as a source to extract an approximately 4.2 kd "core" polypeptide, amyloid plaque core protein (APCP), herein referred to as " $\beta$ -amyloid core protein." This peptide was designated  $\beta$ -protein by Glenner, G., et al, [Biochem Biophys Res Commun (1984) 120:885-890]. The amino acid sequence of the amino-terminus has been determined [Glenner, G., et al, Biochem Biophys Res Commun (1984) 122:1131-1135; Masters, C.L., et al, Proc Natl Acad Sci USA (1985) 82:4245-4259] and the amino acid sequences reported by the two groups are identical except that Glenner et al, report a glutamine at position 11 for Alzheimer Disease cerebral vascular amyloid whereas Masters et al, report glutamic acid at position 11. Also, the former authors report that the cerebral vascular amyloid has a unique amino-terminus while the latter authors report that the form found in amyloid plaque cores has a "ragged" amino-terminus -- i.e., peptides isolated from this source appear to be missing 3, 7, or 8 amino acids from the amino-terminus. Both groups have shown that the same peptide is found in the amyloid plaque cores and vascular amyloid of adult Downes syndrome-afflicted individuals and report glutamic acid at position 11.

Further studies on the  $\beta$ -amyloid core protein were also conducted by Roher, A., et al, Proc Natl Acad Sci USA (1986) 83:2662-2666 which showed the complete amino acid composition of the protein, and verified that it matched that of no known protein. The compositions obtained were, however, evidently not in agreement with those of Allsop, D., et al, Brain Res (1983) 259:348352; nor were they in agreement with those published by Glenner or Masters (supra).

Wong, C.W., et al, Proc Natl Acad Sci USA (1985) 82:8729-8732 showed that a synthetic peptide

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which was homologous to the first ten amino acids of the  $\beta$ -amyloid core protein described by Masters (supra) was able to raise antibodies in mice and that these antibodies could be used to stain not only amyloid-laden cerebral vessels, but neuritic plaques as well. These results were confirmed by Allsop, D. et al, Neuroscience Letters (1986) 68:252-256 using monoclonal antibodies directed against a synthetic peptide corresponding to amino acids 8-17. Thus, in general, the plaque protein found in various locations of the brain of Alzheimer's patients appears to be similar in immunoreactivity. It is highly insoluble, as shown by the inability to achieve solubilization in many commonly used denaturants such as detergents and chaotropic agents (Masters, supra, Allsop, D., et al, (supra)).

It is believed, by analogy to some other amyloid proteins, that  $\beta$ -amyloid core protein may be formed from a precursor in the peripheral circulatory system or lymphatic system. There are six known instances of disease-associated amyloid deposits in which the nature of the precursor protein for the amyloid protein is known: for primary amyloidosis, the source is an immunoglobulin light chain; for secondary amyloidosis, the precursor is amyloid A protein; for familial amyloid polyneuropathy and senile cardiac amyloidosis, prealbumin or a variant thereof; for medullary carcinoma of thyroid, a procalcitonin fragment; and for hereditary cerebral hemorrhage, gamma-trace fragment (See, e.g., Glenner, G. New England Journal of Medicine (1980) 302:1283; Sletton, K., et al, Biochem J (1981) 195:561; Benditt, et al, FEBS Lett (1971) 19:169; Sletton, K., et al, Eur J Biochem (1974) 41:117; Sletton, K., et al, J Exp Med (1976) 143:993). The foregoing is a partial list and there are at least a number of additional references with regard to

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procalcitonin fragment as a precursor for the amyloid of the thyroid carcinoma. Alternatively, or additionally, such a precursor for  $\beta$ -amyloid core protein may be produced in the brain.

5           It has been described that a protein containing the  $\beta$ -amyloid core protein sequence within the framework of a larger protein exists (Kang, J., et al, Nature (1987) 325:733-736). This protein, which is a potential precursor in vivo to the  $\beta$ -amyloid core  
10 protein, was predicted from the sequence of a cDNA clone isolated from a human fetal brain tissue cDNA library and consists of 695 amino acid residues wherein the amino terminus of the  $\beta$ -amyloid core protein begins at position 597. By analogy to the above described series,  
15 it may be that such a precursor or a fragment thereof circulates in the serum at a level differentiable in Alzheimer's victims relative to unafflicted individuals. Alternatively or additionally, such differences may be detected in the cerebral spinal fluid.

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#### Disclosure of the Invention

It is one general object of the invention to provide DNA sequence and protein compositions for  $\beta$ -amyloid-related proteins which can be used for  
25 improved screening, diagnosis, characterization, and study of the etiology of Alzheimer's disease.

In particular the invention provides DNA sequences which represent a cDNA encoding a complete peptide sequence of a novel  $\beta$ -amyloid-related protein,  
30 useful for the preparation of  $\beta$ -amyloid core protein or  $\beta$ -amyloid-related proteins, or for the preparation of nucleic acid reagents.

In a preferred embodiment of this aspect of the invention is provided a DNA sequence wherein a  
35 subfragment of the sequence shown in Figure 1

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corresponds to a 168 basepair insert fragment of the  $\beta$ -amyloid-related gene product of bacteriophage  $\lambda$ APCP168i4.

5 In another aspect of the invention one or more synthetic peptides or recombinantly produced proteins, exclusive of the 28 amino-terminal residues encoding the  $\beta$ -amyloid core protein, containing an immunogenic portion of the  $\beta$ -amyloid core protein or  $\beta$ -amyloid-related proteins are provided and used to  
10 prepare antibodies specific against  $\beta$ -amyloid core protein or  $\beta$ -amyloid-related proteins. These antibodies are reactive with such proteins present at a differentiable level in individuals with Alzheimer's disease, relative to normal, unafflicted individuals.  
15 Such antibodies are useful in diagnostic assays for the disease.

A further aspect of the invention relates to the use of DNA sequences encoding the  $\beta$ -amyloid core protein or  $\beta$ -amyloid-related proteins to prepare  
20 expression vectors which enable the production of such proteins free from contaminating proteins normally accompanying such proteins in vivo. These proteins can be used to produce reagents for diagnostic assays for Alzheimer's disease.

25 Yet another aspect of the invention provides a method for producing the novel protease inhibitor from a transformed microorganism as a secreted, properly folded protein, which method comprises:

30 a) constructing a chimeric DNA sequence encoding a signal peptide sequence fused in phase with the protease inhibitor sequence;

b) expressing the chimeric DNA sequence in a bacterial host strain capable of secreting the protein into the culture medium, which host has been transformed  
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with a vector capable of expressing the chimeric DNA sequence; and

c) recovering the secreted, properly folded protein.

5

Also provided is a method for recovering the refolded, substantially purified protease inhibitor from a transformed microorganism containing a DNA sequence encoding the protease inhibitor, which method comprises:

10

a) harvesting the bacterial broth from the transformed, cultured microorganism;

b) separating the soluble material containing the protease inhibitor from the bacterial cell;

15

c) reacting the soluble material with an affinity matrix to which a serine protease is bound to capture the protease inhibitor;

d) eluting the captured protease inhibitor from said matrix under acidic conditions; and

20

e) purifying the protease inhibitor in the eluate by high performance liquid chromatography to recover the refolded, substantially purified protease inhibitor.

25

Providing improved methods of screening for and diagnosing Alzheimer's disease which use the DNA sequences, proteins, and peptides derived therefrom, are additional aspects of the invention.

30

Thus, another aspect of the invention provides both prognostic and diagnostic methods designed for determining a genetic predisposition to Alzheimer's disease in a test subject. Initially, the invention DNA sequences are used as standards to identify one or more mutations in the  $\beta$ -amyloid protein gene region which are associated with a predisposition to Alzheimer's disease. A probe which spans this mutation can differentiate normal unmutated DNA or RNA from mutated DNA or RNA,

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providing a method of genetic screening for the disease predisposition.

One region of interest for use in a diagnostic assay centers on a 168 basepair (bp) DNA sequence found in  $\lambda$ APCP168i4, a  $\beta$ -amyloid-related cDNA sequence whose structure is provided herein. Oligonucleotide probes specific for this 168 basepair region and for the junction of the corresponding nucleotide region lacking this region are used to confirm the lack of identity between this gene product and the  $\beta$ -amyloid-related species described by Kang et al. The availability of this sequence permits the design of oligonucleotides and oligopeptides which are specific to two distinct, but closely related  $\beta$ -amyloid-related mRNAs and proteins.

In a related prognostic test, a labeled probe derived from  $\beta$ -amyloid sequences is used in identifying one or more restriction site alterations in the region of the  $\beta$ -amyloid gene which are associated with a predisposition to Alzheimer's disease. The labeled probe is hybridized with size-fractionated restriction fragments from a test-subject genomic DNA sample, e.g., by Southern blotting, to identify restriction site alterations which are associated with a predisposition to the disease.

Yet another aspect of the invention relates to the construction and expression of a 57 amino acid protease inhibitor.

Improved therapeutic applications for the treatment of Alzheimer's disease are provided by another aspect of the invention. This application is based on the protein sequence predicted from the sequence of the aforementioned 168 bp insert found in  $\lambda$ APCP168i4. The comparison of this insert protein sequence to the sequences of other known proteins which are well characterized as basic serine protease inhibitors

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indicates that the  $\lambda$ APCP168i4 insert protein sequence will possess the functional properties associated with basic protease inhibitors. Thus, this particular protein sequence can be used as a therapeutic to prevent the formation of neuritic plaques.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

#### Brief Description of the Drawings

Figure 1 shows the base sequence of a cDNA clone, designated  $\lambda$ APCP168i4, which encodes amino acids 1-751 of  $\beta$ -amyloid-related protein. The 168 bp insert, which distinguishes this clone from the Kang et al sequence, is underlined.

Figure 2 shows a DNA sequence of a genomic clone encoding the first 18 amino acids of the  $\beta$ -amyloid core protein as described by Masters et al. It also encodes, immediately preceding these amino acids, a methionine codon which could potentially be used as an initiating codon;

Figure 3 shows the base sequence of a cDNA clone, designated  $\lambda$ SM2W4, whose 3' end encodes the first four amino acids of  $\beta$ -amyloid core protein. It also encodes, immediately preceding these amino acids, a methionine codon as described above;

Figure 4 shows the base sequence of a cDNA clone, designated  $\lambda$ SM2W3, which encodes 97 amino acids; the first 26 of these correspond to the region of the  $\beta$ -amyloid core protein described by Masters et al, from Glu<sub>3</sub> through Ala<sub>28</sub>;

Figure 5 shows the base sequence and corresponding amino acid sequence of a  $\beta$ -amyloid-related protein deduced from  $\lambda$ SM2W4 and  $\lambda$ SM2W3;

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Figure 6 shows the nucleotide and deduced amino acid sequence of the  $\lambda$ SM2W9  $\beta$ -amyloid clone;

Figure 7 shows a comparison of the sequences of  $\lambda$ SM2W3 and  $\lambda$ SM2W9;

5        Figure 8 shows the detection of mRNAs for  $\lambda$ APCP168i4 and the mRNA described by Kang et al on a Northern blot produced using RNA's isolated from human brain and human cells in culture and hybridized to oligonucleotide probes which are specific for each  
10        species;

Figure 9 shows the construction scheme for a bacterial expression vector for the production of a  $\beta$ -amyloid-related protein in bacteria;

15        Figure 10 shows the construction scheme for a recombinant vaccinia virus expression vector for the expression of the protein encoded by  $\lambda$ APCP168i4;

Figure 11 shows the construction scheme for a mammalian cell expression vector for the expression of the protein encoded by  $\lambda$ APCP168i4;

20        Figure 12 shows the construction of an expression vector for the production of the  $\beta$ -amyloid-related protein described in Figure 5, when the methionine encoded immediately upstream from the  $\beta$ -amyloid core protein sequence is used as an initiating  
25        methionine;

Figure 13 shows the relatedness of the peptide encoded by the  $\lambda$ APCP168i4 168 bp insert to a superfamily of proteins many of whose members exhibit inhibitory activity for basic proteases; and

30        Figure 14 shows the construction of a synthetic tryptophan operon promoter and operator regulatory sequence, and a restriction site map of plasmid pTRP233.

35        Figure 15 shows the results of a Western blot analysis of the CV-1 cells producing the 751 amino acid

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$\beta$ -amyloid protein using  $\beta$ -amyloid specific polyclonal antisera. The control is the pSC11 vaccinia virus lacking the  $\beta$ -amyloid coding sequence.

Figure 16 is an illustration of the  
5 oligonucleotide sequences used to construct chimeric genes containing either the ompA signal sequence fused to the protease inhibitor sequence (FIG. 16A) or the phoA signal sequence fused to the protease inhibitor  
10 sequence (FIG. 16B). The asterisks indicate the individual oligonucleotides used for each construction.

### Detailed Description of the Invention

#### A. Definitions

15 As used herein, " $\beta$ -amyloid core protein" means the protein described by Masters, C.L., et al Proc Natl Acad Sci USA (1985) 82:4245-4249, herein referred to as "Masters, et al". This approximately 4 kD protein is defined at the amino terminus by sequence analysis as a  
20 mixture of four peptides with slightly different amino termini, the amino termini of the three smaller peptides being completely encoded by that of the largest. The first 28 amino acids of the longest form is  
25 Asp<sub>1</sub>-Ala<sub>2</sub>-Glu<sub>3</sub>-Phe<sub>4</sub>-Arg<sub>5</sub>-His<sub>6</sub>-Asp<sub>7</sub>-Ser<sub>8</sub>-Gly<sub>9</sub>-Tyr<sub>10</sub>-Glu<sub>11</sub>-Val<sub>12</sub>-His<sub>13</sub>-His<sub>14</sub>-Gln<sub>15</sub>-Lys<sub>16</sub>-Leu<sub>17</sub>-Val<sub>18</sub>-Phe<sub>19</sub>-Phe<sub>20</sub>-Ala<sub>21</sub>-Glu<sub>22</sub>-Asp<sub>23</sub>-Val<sub>24</sub>-Gly<sub>25</sub>-Ser<sub>26</sub>-Ser<sub>27</sub>-Ala<sub>28</sub>.

" $\beta$ -amyloid-related protein or " $\beta$ -amyloid-related peptide" are defined herein as those proteins containing within their sequence the  $\beta$ -amyloid core  
30 protein sequence defined above or fragments of such proteins which do not necessarily include the  $\beta$ -amyloid core protein sequence as defined above. As an example, this term is used to refer to the protein described by Kang, J. et al, Nature (1987) 325:733-736, herein  
35 referred to as "Kang, et al" which contains the

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β-amyloid core protein within its structure at amino acid 597 of a 695 amino acid protein. As another example, it refers to the protein encoded by λAPCP168i4, shown in Figure 1, which, within its structure, contains the β-amyloid core protein at amino acid 653 of a 751 amino acid protein.

"Immunogenic β-amyloid core peptide" or "immunogenic β-amyloid-related peptide" refer to peptides whose amino acid sequences match those of some region of the β-amyloid core protein or β-amyloid-related protein, and which are capable of provoking an antibody response in an immunized animal.

"Genetic predisposition to Alzheimer's disease" refers to an identifiable genetic mutation or alteration found in the genomes of individual's with Alzheimer's disease, or those individuals destined to develop Alzheimer's disease, but not normal (nondiseased) individuals.

#### B. DNA Sequences

DNAs corresponding to β-amyloid core protein or β-amyloid-related protein sequences are useful as probes in diagnosis. Several DNAs containing sequences encoding portions of β-amyloid-related protein sequence, and β-amyloid core protein sequence with adjacent noncoding segments are disclosed herein. These DNA sequences in whole or in part, are thus useful in diagnosis, either as intact probes, or as fragments.

In particular, the invention includes a DNA sequence which encodes a β-amyloid-related protein comprising the nucleotide sequence and corresponding, deduced amino acid sequence set forth in Figure 1. This DNA sequence encodes an approximately 82,610 dalton protein containing the β-amyloid-related core protein.

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The present  $\beta$ -amyloid protein cDNA sequence, set forth in Figure 1, can be isolated from bacteriophage  $\lambda$ APCP168i4. This human fibroblast cDNA clone was obtained from a cDNA library prepared in  $\lambda$ gt-10 using standard techniques from SV40-transformed fibroblast (SV80) cells (Todaro, G.J., et al, Science (1966) 153:1252-1254). The  $\lambda$ gt10-SV80 library was screened with a mixture of labelled oligonucleotides. Two unique phage containing  $\beta$ -amyloid-related sequences were obtained; these  $\beta$ -amyloid-related sequences were subcloned into a plasmid vector and sequencing analysis revealed a sequence co-linear with the sequence encoding the Kang et al  $\beta$ -amyloid-related protein, except for the presence of a 168 basepair insert. The 168 basepair insert interrupts the codon for Val289 of the Kang et al sequence, resulting in the loss of this amino acid from the  $\lambda$ APCP168i4 protein. The 168 basepair insert, together with the 3 basepairs gained from the interrupted Val289 codon, encode 57 new amino acids, which are underlined in Figure 1. Downstream of this insertion, at codon 653 of Figure 1, lies the amino-terminal aspartate of the  $\beta$ -amyloid core protein described by Masters et al. The  $\lambda$ APCP168i4 clone was deposited at ATCC on 1 July 1987 under the accession number 40347.

Particularly useful are those sequences which encode the 57 amino acid insert found in  $\lambda$ APCP168i4, as well as sequences encoding the corresponding "junction" of the Kang et al  $\beta$ -amyloid-related protein sequence.

For example, one preferred embodiment comprises DNA sequences encoding a  $\beta$ -amyloid-related protein having an amino acid sequence corresponding to residues 289 through 345 of the above-identified protein. Thus, this embodiment comprises a  $\beta$ -amyloid-related protein of the amino acid sequence:

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Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala  
 10  
 Met Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
 5 20  
 Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn  
 30 40  
 Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala  
 50  
 10 Ile.

This particular peptide, including any  
 fragments thereof, distinguishes the present  
 $\beta$ -amyloid-related protein from other reported forms.

15 In another preferred embodiment, the invention  
 discloses a  $\beta$ -amyloid-related protein having the D4A  
 sequence and deduced amino acid sequence corresponding  
 to amino acid residues 284-Val<sub>289</sub>-(V289-345)-349 of the  
 $\beta$ -amyloid-related sequence set forth in Figure 1  
 20 (wherein V symbolizes a deletion of residues 289 through  
 345). An oligopeptide spanning this specific region  
 would be useful to generate a protein specific  
 diagnostic reagent to differentiate between the  
 $\beta$ -amyloid-related protein genetic variant described by  
 25 Kang et al and the  $\beta$ -amyloid-related protein of the  
 present invention. Thus, this embodiment comprises a  
 $\beta$ -amyloid-related protein of the amino acid sequence:

Glu Glu Val Val Arg Val Pro Thr Thr Ala  
 30 5 10

A smaller peptide contained within the sequence of this  
 peptide might also be used.

35 Oligonucleotides specific for the 168 basepair  
 insert and for the junctions of this region of the



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5  $\beta$ -amyloid-related protein described by Kang et al can be  
 synthesized and used to compare the levels of mRNA  
 expression of these two distinct proteins. As an  
 example, oligonucleotides specific for the 168 basepair  
 insert, designated oligo #2734

10 (CGCCGTA<sup>10</sup>AAA GAATGGGG<sup>20</sup>CA CACTTCCCT<sup>30</sup>T CAGTCACATC<sup>40</sup> AAAGTACCAG<sup>50</sup>  
 10 CGGGAGATCA<sup>60</sup>)

and for the "junction" region, designated oligo #2733

15 (CTGCTGT<sup>10</sup>TGT AGGAACTCGA<sup>20</sup> ACCACCTCT<sup>30</sup>T)

were synthesized using phosphoramidite chemistry on an Applied Biosystems DNA synthesizer.

20 The "junction" oligo is complementary to 15  
 basepairs on either side of the insert and is used to  
 distinguish between the published  $\beta$ -amyloid-related  
 protein sequences and the  $\lambda$ APCP168i4 sequences by  
 specific hybridization conditions known in the art under  
 which a 15 basepair perfect match is unstable, while a  
 30 basepair perfect match is stable. These  
 25 oligonucleotides are used to screen cDNA libraries or  
 mRNA from various sources as an assay for measuring the  
 level of expression of a specific sequence.

30 Another example, described below, is a genomic  
 sequence encoding the first 18 amino acids (19 if Met is  
 included) of the  $\beta$ -amyloid protein sequence  
 characteristic of Alzheimer's disease in neuritic  
 plaques. The clone was obtained in  $\lambda$  Charon 4A from the  
 genomic library described by Lawn, R.M., et al, Cell  
 (1978) 15:1157-1174 and has been partially sequenced, as  
 35 shown in Figure 2. As seen, the sequenced portion of  
 the genomic clone includes a 57 base pair segment which

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encodes the amino acids 1-18 of the previously reported  $\beta$ -amyloid core protein and a methionine immediately preceeding. Downstream of the amino acid 18 codon, the genomic sequence diverges in codon sequence from that expected from the reported amino acid sequence of  $\beta$ -amyloid core protein. By reference to the protein encoded by the sequence of Figure 4, and by inspection of the sequences flanking this region using knowledge known in the art, this divergence is likely to be an intron sequence. This clone, designated  $\lambda$ SM2, was deposited at ATCC on 13 November 1986.

A HindIII/RsaI probe derived from the genomic clone (see Figure 2) was used as a probe to isolate, according to standard procedures, cDNA fragments from a cDNA library constructed in  $\lambda$ gt10 from temporal and parietal cortical tissue of a normal human brain (the individual was a 55 year old man who died of myocardial infarction). The three cDNA clones which were isolated were sequenced conventionally, and matched with amino acid sequences in each of the three possible reading frames to identify regions coding for  $\beta$ -amyloid-related proteins. One of the clones, designated  $\lambda$ SM2W4, contains a 3'-end terminal sequence which encodes the Asp Ala Glu Phe amino acids at the 5'-end of  $\beta$ -amyloid-core protein, as seen in Figure 3, which shows the complete base sequence of the clone. The Asp1 codon is immediately preceeded by a methionine codon. A second clone, designated  $\lambda$ SM2W3, contains a 5' region segment which has a 6 bp overlap with the 3' end of the  $\lambda$ SM2W4 clone (an EcoRI restriction site), encoding amino acids 3 and 4 of the  $\beta$ -amyloid core protein, and an additional 95 codons which encode the remainder of a  $\beta$ -amyloid-related protein. The DNA sequence for the 100 amino acid protein (including Met) encoded in  $\lambda$ SM2W4 and  $\lambda$ SM2W3 is shown in Figure 5. It is, of course,

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understood that the methionine is probably processed in vivo, and that the  $\beta$ -amyloid-related protein represented in this figure may thus be a 99 amino acid sequence.

5 A third cDNA clone encodes a portion of a  $\beta$ -amyloid-related protein which differs from  $\lambda$ SM2W3 in the region shown by 15 nucleotide differences and 4 amino acid differences in the region of amino acids 3-44 of Figure 5. The DNA sequence and deduced amino acid sequence for this clone, designated  $\lambda$ SM2W9 are given in  
10 Figure 6. A comparison with  $\lambda$ SM2W3 is given in Figure 7.

### C. Protein Production

15 The cDNA clones described herein permit construction of coding sequences which may be expressed to obtain a complete  $\beta$ -amyloid-related protein, an 100 amino acid  $\beta$ -amyloid-related protein containing the amino-terminal sequences reported for  $\beta$ -amyloid core protein, and other desired proteins. These sequences  
20 can be inserted in a suitable expression vector for production of protein. Details of the method of constructing a DNA subsequence of Figure 1 and insertion of this sequence into a bacterial expression vector is provided in Example 2.

25 Briefly, an E. coli expression vector, designated pAPCP118-3, was constructed for the expression of a fusion protein consisting of amino acid residues 655 to 751 set forth in Figure 1. The construction of pAPCP118-3 was accomplished by joining  
30 the following three fragments: (1) a plasmid backbone (consisting of pBR322 replication functions, an ampicillin resistance gene, the tryptophan promoter and operator, a ribosome binding site, DNA encoding the seven amino terminal codons of the  $\beta$ -galactosidase  
35 structural gene followed by six threonine residues, and

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transcription termination signals: (2) an EcoRI-HaeII fragment encoding amino acid residues 655-728 of the Figure 1 sequence; and (3) a synthetic fragment encoding amino acid residues 729-751 of the Figure 1 sequence, followed by a stop codon.

The resulting vector was used to transform E. coli W3110 and expression of the fusion protein was induced by reducing the tryptophan concentration followed by the addition of 3- $\beta$ -indoleacrylic acid. The resulting protein can be purified using conventional purification techniques and the resulting purified material is available for use in the production of antibodies for diagnostic assays.

The complete coding sequence of the  $\beta$ -amyloid-related protein set forth in Figure 1 was subcloned in two fragments from the deposited  $\lambda$ APCP168i4 clone and inserted into pSC11, a vaccinia virus expression vector. The construction of the resulting vector, pFL4T4BV, is illustrated in Figure 10. Briefly, an approximately 1.06 kilobase (kb) EcoRI fragment, spanning amino acid residues 655-751 of the protein illustrated in Figure 1, was cloned into EcoRI-digested plasmid pGEM-3<sup>TM</sup> (available from Promega Biotec) to create an intermediate vector designated p4BI. Subsequently p4BI was digested with HindIII to remove much of the 3'-noncoding sequence of the  $\beta$ -amyloid-related sequence. The resulting vector p4BARI was digested with EcoRI and treated with calf intestinal alkaline phosphatase prior to ligation to the 2088 bp EcoRI fragment derived from  $\lambda$ APCP168i4 to form p4T4B. This plasmid was digested with SmaI and XmnI to generate a 2678 bp fragment spanning the complete protein encoding sequence set forth in Figure 1.

The gene encoded by this SmaI-XmnI fragment was inserted into a well-known vaccinia viral vector,

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pSC11, for subsequent expression of the  $\beta$ -amyloid-related protein in C7-1 monkey kidney cells using a eucaryotic transient expression system as described by Cochran, M.A., et al, Proc Natl Acad Sci USA (1985) 82:19-23. More commonly, this vector is used for in vivo protein and antibody production in animals after its sequences have been inserted into the vaccinia virus genome (see "Antibody Production" section below).

Similarly, mammalian vectors can be utilized for expression of the  $\beta$ -amyloid core protein or  $\beta$ -amyloid-related proteins described herein. For example, plasmid pHGH-SV (10) (a plasmid described in EPA 217,822, published 15 April 1987, and incorporated herein by reference) contains a pUC8 plasmid backbone, hMT-IIa gene promoter and regulator elements, SV-40 DNA promoter and enhancer elements, and the coding portions of the hGH gene and 3' regulatory sequences. This plasmid can be digested with BamHI and SmaI and treated with BamHI linkers to delete the human growth hormone protein encoding sequence and leaving the 3'-noncoding sequences and regulatory elements attached to the plasmid backbone. This approximately 5100 base pair DNA piece is gel purified and ligated to BamHI linkers. Digestion with BamHI, repurification of the DNA fragment and subsequent ligation result in a plasmid designated pMTSV40 polyA Bam which contains the structural and regulatory elements comprising a mammalian cell expression vector. After BamHI digestion of pMTSV40 polyA BamHI and repair in the presence of DNA polymerase I and all four dNTPs, this vector is available for insertion of the ~ 2678 bp SmaI-XmnI restriction fragment of plasmid p4T4B. The resulting vector can then be used for efficient protein expression in CHO cells as described in Example 4.

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In addition, the sequence information from the  $\lambda$ SM2W4 clone, illustrated in Figure 3, combined with the sequences present in the  $\lambda$ SM2W3 clone, may be used to construct a mammalian cell expression vector encoding the protein described in Figure 5.

The secreted protease inhibitor may be recovered in a biologically active, refolded and substantially pure form from the bacterial broth using a solid support affinity matrix, such as, for example, Sepharose beads, to which a serine protease with high affinity for the inhibitor activity is bound. Enzymes available for this use include, for example, the human serine proteases trypsin and chymotrypsin. Once the protease inhibitor is captured on the beads, the protein may be eluted using acid conditions, such as a low pH environment in the range of about 1.0 to about 5.0, preferably 1.25. The eluted protein may be substantially purified, i.e., recovered at least 70%, preferably 80%, more preferably 90%, most preferably at least 95%, as measured by high performance liquid chromatography (HPLC) (e.g., a C4 column using a 60% acetonitrile 0.1%/trifluoroacetic acid elution gradient).

#### D. Antibody Preparation

Antibodies specific against  $\beta$ -amyloid core protein and  $\beta$ -amyloid-related proteins are prepared by known procedures. As an example using synthetic peptides, typically the protein sequence is analysed for regions of at least about 10 amino acids long which have predominantly polar and/or charged amino acid residues to identify favorable immunogenic regions.

As another example, the DNA sequence shown in Figure 1 can be used to design oligopeptides which are specific to the inserted sequence in  $\lambda$ APCP168i4, as well as the corresponding junction of this insert to the

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5  $\beta$ -amyloid-related protein described by Kang et al. For example, an oligopeptide spanning the inserted junction such as Glu-Glu-Val-Val-Arg-Val-Pro-Thr-Thr-Ala may be used to immunize animals to produce a specific antisera against this region of the protein described by Kang et al. Inspection of the Kang et al sequence in the absence of knowledge of the  $\lambda$ APCP168i4 sequence would not provide the information necessary to identify this peptide as a valuable reagent by any method known in the art. As another example, oligopeptides designed to represent sequences present in the 168 basepair insert region could be used in a similar manner to generate antisera against this unique region of the APCP168i4 protein. Thus, the regions identified as favorable for immunogenicity are synthesized by conventional peptide synthetic methods, and coupled covalently to a suitable carrier protein, such as keyhole limpet hemocyanin. Antibodies are raised against the peptide/protein conjugate in rabbits or the like by conventional methods. The presence of antibody in immunized animals is detected by standard methods, such as immunoreactivity to the immunizing synthetic peptide affixed to a microtiter plate, followed by ELISA.

25 Another method of antibody production uses the bacterially produced  $\beta$ -amyloid-related fusion protein of Example 2 as the immunogen. The immunogenicity of this protein is shown by the immunoreactivity of the antisera to the bacterially produced fusion protein.

30 Yet another method of antibody production relies on the inoculation of the host animal with a live recombinant vaccinia virus encoding  $\beta$ -amyloid-related protein, such recombinant viruses being generated by established techniques involving recombination between wild-type vaccinia virus and the vectors derived from pSC11, such as pFL4T4BV, described herein. These

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antibodies can then be used in the diagnostic assays described below.

5 A panel of antibodies which are specific against peptides derived from different regions of the  $\beta$ -amyloid-related protein, such as the 57 amino acid insert of  $\lambda$ APCP163i4, are further analysed for immunoreactivity of  $\beta$ -amyloid-related proteins present in the serum or cerebral spinal fluid of patients with Alzheimer's disease, to identify antibodies suitable for a diagnostic assay for Alzheimer's disease, as discussed below.

#### E. Diagnostic and Prognostic Methods

15 The DNA sequences described in Figures 3, 4, and 6 for  $\beta$ -amyloid-related protein are primarily derived from an apparently normal advanced-age male showing no signs of Alzheimer's disease at the time of death. The  $\lambda$ APCP168i4 sequence described in Figure 1 for another  $\beta$ -amyloid-related protein is derived from cultured fibroblast cells. These sequences provide a standard for identifying mutations in genomic sequences which are found in individuals with Alzheimer's disease, and which are therefore likely to be associated with a predisposition to the disease.

25 1. Prognostic Methods. Assays are used to determine an individual's genetic predisposition to Alzheimer's disease. These tests use the DNA sequences of the present invention in a comparative study with samples of the patient's DNA to define polymorphisms in the region of the chromosome containing the  $\beta$ -amyloid gene. Alternatively or concurrently, the DNA sequences of the present invention can be used in nucleic acid hybridization analysis to define alterations, which alterations are meant to include additions, deletions,

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mutations or substitutions, in the DNA or RNA encoding  $\beta$ -amyloid-related proteins.

Alterations in the  $\beta$ -amyloid-related protein sequences which correlate with Alzheimer's disease can be assayed by a differential probe binding method. Under appropriate hybridization conditions, known in the art, the oligonucleotide probes will bind to completely complementary sequences, but not to closely related but altered sequences.

In one assay method, nucleic acid samples prepared from the test subject are hybridized with each probe, under the defined hybridization conditions, and examined for binding to specific oligonucleotides. Alterations, and thus predisposition to Alzheimer disease, are confirmed by binding one probe, but not to the other probe. The probe-binding method, as it has been applied to other genetic diseases, is described in Conner, B.J., et al, Proc Nat Acad Sci (USA) (1983) 80:278-282.

Alternatively, probes derived from the genomic or cDNA sequences described above may be used to identify restriction fragment length polymorphisms which are associated with a genetic predisposition to Alzheimer's disease. Initially the probes are used to identify restriction site fragment lengths from both normal and diseased genomic digest samples. Changes in restriction fragment lengths which correlate with Alzheimer's disease are then applied to genetic screening, by standard methods. That is, test subject genomic material is digested with the restriction enzyme(s) of interest, and the fragment pattern on Southern blotting is determined with the labeled probe.

2. Diagnostic Methods. In various other clinical amyloidoses, the amyloidogenic peptides are variants of normally expressed gene products. These

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peptides have been altered either by aberrant proteolytic processing or by genetic lesions yielding an alteration in the primary amino acid sequences. There are known amyloidosis, such as Familial Amyloid Polyneuropathy (FAP), in which a mixture of the normal precursor and the amyloidogenic variant coexist within the circulation. An aberrant tissue-distribution for the expression of the aberrant gene product, or some other alteration in its level of expression, its sequence, or its processing in Alzheimer's disease could have significance in terms of the etiology of amyloid deposition.

A first diagnostic test which utilizes the materials of the invention is a direct antibody assay for the increase or decrease of  $\beta$ -amyloid core protein or  $\beta$ -amyloid-related proteins in Alzheimer's individuals relative to normal individuals. In this method, antibodies obtained as described above are screened for specific immunoreactivity with proteins from individuals known to have Alzheimer's disease. The presence of immunoreactive serum proteins is determined by standard immunoassay techniques, such as solid-phase ELISA techniques.

The body sample which is assayed for the presence of  $\beta$ -amyloid core protein or  $\beta$ -amyloid-related protein is, for example, serum or cerebral spinal fluid. For instance, in hereditary cerebral hemorrhage with amyloidosis, a disorder wherein the amyloid is generated from the gamma-trace precursor, the precursor can be detected in cerebrospinal fluid using an immunoassay. The levels of the precursor are reduced in the patients having the disease, leading to the conclusion that it is used up during the formation of the deposits. The precursor is made in the brain, and hence the cerebrospinal fluid is the appropriate sample.

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5 In another diagnostic test, DNA encoding  $\beta$ -amyloid-related protein is directly useful as a probe to detect an increase or decrease in synthesis of mRNAs encoding  $\beta$ -amyloid-related proteins in the appropriate target cells by virtue of its ability to hybridize to the appropriate mRNA. An example showing the utility of this method is given in Example 5 below.

10 A third diagnostic assay permits the detection of antibodies against the amyloid protein in patient's serum using such standard ELISA techniques wherein the purified recombinant amyloid protein or synthetic peptide is bound to the solid support.

15 F. Therapeutic Methods.

The invention also provides for improved therapeutic treatments for Alzheimer's disease. One therapeutic treatment is suggested by the sequence of the protein encoded by the 168 bp insert in  $\lambda$ APCP168i4. Using methods well known in the art such as the use of computer programs which search protein databases, to compare the protein relatedness of one protein to another, the protein encoded by the 168 bp insert was found to be homologous to a family of proteins known as Kunitz-type basic protease inhibitors. The level of relatedness of the insert protein segment to three members of the Kunitz family is shown in Figure 13, where the symbol (:) indicates an identity between the two sequences compared and the symbol (.) indicates the substitution of an amino acid with similar chemical properties. The insert sequence, depicted by the one-letter amino acid code as EVCS ... GSAI is shown to be related to a high degree over its entire length to all members of the Kunitz family (only three are shown as an example). The comparisons shown are to: (1) a human trypsin inhibitor, a secreted plasma protein which

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inhibits trypsin, plasmin and lysosomal granulocytic elastase (Wachter, E., and Hochstrasser, K. Hoppe-Seyler's Z Physiol Chem (1981) 362:1351-1355; Morii, M., and Travis, J. Biol Chem Hoppe-Seyler (1985) 366:19-21; (2) a bovine trypsin inhibitor which inhibits trypsin, chymotrypsin, elastases and plasmin (Hochstrasser, K. and Wachter, E., Hoppe-Seyler's Z Physiol Chem (1983) 364:1679-1687; Hochstrasser, K., et al, Hoppe-Seyler's Z Physiol Chem (1983) 364:1689-1696; and (3) a bovine serum basic protease inhibitor (and its precursor) which inhibits trypsin, kallikrein, chymotrypsin, and plasmin (Anderson, S. and Kingston, I.B. Proc Nat Acad Sci (USA) (1983) 80:6838-6842. Based on this level of relatedness to the 168 bp insert protein sequence, one interpretation is that this region of the  $\lambda$ APCP168i4 protein has a function as a protease inhibitor in vivo.

This inhibitor may be used to ameliorate or prevent Alzheimer's disease in a number of ways. Since the Alzheimer's disease-associated amyloid plaques result from proteolysis of the  $\beta$ -amyloid precursor protein, administration of the inhibitor to prevent cleavage of the  $\beta$ -amyloid precursor is likely to prevent plaque formation. While not wishing to be bound by this interpretation, an alternative mechanism of action for the inhibitor may involve its role in the inhibition of a protease which degrades the plaque. Administration of an antagonist, for example a specific antibody, either monoclonal or polyclonal, that is reactive to the inhibitor and able to block the interaction of the inhibitor to the protease is therapeutically useful.

This or other protease inhibitors, peptidic or non-peptidic, could be used to treat or prevent Alzheimer's disease by a mechanism such as preventing the formation of neuritic plaques. One method of

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administration might involve nasal delivery of such a peptide (as the blood-brain barrier is known to be more open immediately behind the nasal cavity). Nasal delivery could be accomplished by formulating the protease inhibitor peptide with excipient and an effective amount of an adjuvant, such as the fusidic acid derivatives or a polyoxyethylene ether at a concentration of 0.1-10% (w/w). Stabilizers or disinfectants could optionally be added. The amount of peptide would vary, depending on its efficacy and bioavailability, but could range from 0.1-25% (w/w). Administration would occur by spraying from 10-100  $\mu$ l of the solution into each side of the nose from 1-4 times a day, although dosing could also be more or less frequent. Other modes of delivery include a solution of inhibitor in a pharmaceutically acceptable excipient where the inhibitor is 0.1-25% (w/w) and where the inhibitor is administered by injection into the bloodstream or into the spinal column, or directly onto the brain. If the inhibitor is non-peptidic, oral dosing may be possible.

Other utilities of the present protease inhibitor have applicability outside the treatment of Alzheimer's disease. For example, in acute pancreatitis, there is a general release of digestive proteases such as trypsin, chymotrypsin and elastase, from the pancreas into the circulation. It would be useful in the clinical management of this disease to administer one or more protease inhibitors systemically to inactivate these proteases. For example, aprotinin, a protease inhibitor sharing approximately 50% amino acid homology with the present A4<sub>i</sub> inhibitor, has been found to have clinical utility in animal models (H. Fritz and G. Wunderer, Drug Res 33(I), No. 4 (1983) pp. 479-494). The present inhibitor is preferred for this use since it

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is present in low levels in the circulation in the form of its larger precursor so that an allergic or immune reaction would not occur as might be expected with aprotinin or other inhibitors of non-human origin.

5 Yet other forms of the present inhibitor are provided herein. Such analogs of the 57 amino acid protease inhibitor contain at least one amino acid substitution which is effective to yield an inhibitor having altered protease specificity. It is known in the  
10 art that the residue termed P<sub>1</sub> plays a major role in defining the specificity of a protease inhibitor. In the mature secreted inhibitor of the invention, this P<sub>1</sub> residue is Arg<sub>13</sub> which is expected to direct this inhibitor to enzymes having trypsin-like activities. By  
15 analogy to aprotinin, wherein it has been shown that modification to its P<sub>1</sub> residue has modified the protease activity of the inhibitor (see Gebhard, W., et al, in Proteinase Inhibitors, eds. Barrett and Salvesin, Amsterdam, N.Y., Oxford: Elsevier 1986), modification  
20 via site-specific mutagenesis of the present inhibitor produces similar results. For enhanced inhibition of enzymes having chymotrypsin activity, Arg<sub>13</sub> of the present inhibitor is substituted with aromatic amino acids such as, for example, Phe, Tyr and Trp; whereas to  
25 produce an inhibitor having enhanced ability to inhibit enzymes possessing human elastase activity, the Arg<sub>13</sub> residue is substituted with neutral hydrophobic amino acids such as, for example, Leu, Met and Val.

The analogs of the present protease inhibitors  
30 are constructed from oligonucleotides containing the specific codons encoding the desired amino acid at this location, using site-specific mutagenesis techniques as are known in the art. The desired activities of the analogs thus constructed are assayed using the  
35 appropriate enzyme, for example, either trypsin,

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chymotrypsin or elastase as the standard in one of the respective assays using, for example, the trypsin or chymotrypsin assays described in Tan, N.H. Biochem (1977) 16:1531-1541 and the elastase assays Barrett, A.J. (1981) in Methods in Enzymology vol. 80, L. Lorand ed., Academic Press, New York. The activity of the analogs may be compared with that of the natural protease inhibitor of the invention. The kinetics of inhibition ( $K_i$ ) of the natural protease inhibitor for trypsin ( $K_i=3 \times 10^{-9}M$ ) and chymotrypsin ( $8.5 \times 10^{-9}M$ ) are in the nanomolar range and therefore, quite specific.

#### G. Methods and Materials

Most of the techniques which are used to transform cells, construct vectors, extract messenger RNA, prepare cDNA libraries, and the like are widely practiced in the art, and most practitioners are familiar with the standard resource materials which describe specific conditions and procedures. However, for convenience, the following paragraphs may serve as a guideline.

#### Hosts and Control Sequences

Both procaryotic and eucaryotic systems may be used to express the  $\beta$ -amyloid core and  $\beta$ -amyloid-related sequences; procaryotic hosts are, of course, the most convenient for cloning procedures. Procaryotes most frequently are represented by various strains of E. coli; however, other microbial strains may also be used. E. coli strains may secrete the  $\beta$ -amyloid core and  $\beta$ -amyloid-related proteins to the periplasm when the genes encoding these proteins are fused to appropriate signal peptides, and certain E. coli strains, for example, a lipoprotein mutant strain such as JE5505

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(Kanamari, T. Gene (1988) 66:295-300), will excrete the chimeric proteins directly to the culture medium.

Plasmid vectors which contain replication sites, selectable markers and control sequences derived from a species compatible with the host are used; for example, E. coli is typically transformed using derivatives of pBR322, a plasmid derived from an E. coli species by Bolivar, et al, Gene (1977) 2:95. pBR322 contains genes for ampicillin and tetracycline resistance, and thus provides multiple selectable markers which can be either retained or destroyed in constructing the desired vector. Commonly used procaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the  $\beta$ -lactamase (penicillinase) and lactose (lac) promoter systems (Chang, et al, Nature (1977) 198:1056) and the tryptophan (trp) promoter system (Goeddel, et al Nucleic Acids Res (1980) 8:4057) and the lambda derived P<sub>L</sub> promoter and N-gene ribosome binding site (Shimatake, et al, Nature (1981) 292:128).

Other procaryotic control sequences include signal sequences which direct secretion of a protein to the periplasm. Commonly used bacterial signal peptides include the ompA (Kikuchi, et al, Nucleic Acids Res (1981) 9:5671-5678) and phoA (Beck and Bremer, Nucleic Acids Res (1980) 8:3011-3024) signal peptides which can be fused to the protease inhibitor sequence of the invention.

In addition to bacteria, eucaryotic microbes, such as yeast, may also be used as hosts. Laboratory strains of Saccharomyces cerevisiae, Baker's yeast, are most used although a number of other strains or species are commonly available. Vectors employing, for example,



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the 2  $\mu$  origin of replication of Broach, J. R., Meth Enz (1983) 101:307, or other yeast compatible origins of replication (see, for example, Stinchcomb, et al, Nature (1979) 282:39, Tschumper, G., et al, Gene (1980) 10:157 and Clarke, L., et al, Meth Enz (1983) 101:300) may be used. Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess, et al, J Adv Enzyme Reg (1968) 7:149; Holland, et al, Biochemistry (1978) 17:4900). Additional promoters known in the art include the promoter for 3-phosphoglycerate kinase (Hitzeman, et al, J Biol Chem (1980) 255:2073). Other promoters, which have the additional advantage of transcription controlled by growth conditions and/or genetic background are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, the alpha factor system and enzymes responsible for maltose and galactose utilization. It is also believed terminator sequences are desirable at the 3' end of the coding sequences. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes.

It is also, of course, possible to express genes encoding polypeptides in eucaryotic host cell cultures derived from multicellular organisms. See, for example, Axel, et al, U.S. Patent No. 4,399,216. These systems have the additional advantage of the ability to splice out introns and thus can be used directly to express genomic fragments. Useful host cell lines include VERO and HeLa cells, and Chinese hamster ovary (CHO) cells. Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, the commonly used early and late promoters from Simian Virus 40 (SV 40) (Fiers, et al, Nature (1978) 273:113),

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or other viral promoters such as those derived from polyoma, Adenovirus 2, bovine papilloma virus, or avian sarcoma viruses. The controllable promoter, hMTII (Karin, M., et al, Nature (1982) 299:797-802) may also be used. General aspects of mammalian cell host system transformations have been described by Axel (supra). It now appears, also that "enhancer" regions are important in optimizing expression; these are, generally, sequences found upstream or downstream of the promoter region in noncoding DNA regions. Origins of replication may be obtained, if needed, from viral sources. However, integration into the chromosome is a common mechanism for DNA replication in eucaryotes.

#### Transformations

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, S.N., Proc Natl Acad Sci (USA) (1972) 69:2110, or the RbCl<sub>2</sub> method described in Maniatis, et al, Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor Press, p. 254 and Hanahan, D., J Mol Biol (1983) 166:557-580 may be used for procaryotes or other cells which contain substantial cell wall barriers. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology (1978) 52:546, optionally as modified by Wigler, M., et al, Cell (1979) 16:777-785 may be used. Transformations into yeast may be carried out according to the method of Beggs, J.D., Nature (1978) 275:104-109 or of Hinnen, A., et al, Proc Natl Acad Sci (USA) (1978) 75:1929.

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Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

The DNA sequences which form the vectors are available from a number of sources. Backbone vectors and control systems are generally found on available "host" vectors which are used for the bulk of the sequences in construction. For the pertinent coding sequence, initial construction may be, and usually is, a matter of retrieving the appropriate sequences from cDNA or genomic DNA libraries. However, once the sequence is disclosed it is possible to synthesize the entire gene sequence in vitro starting from the individual nucleotide derivatives. The entire gene sequence for genes of sizeable length, e.g., 500-1000 bp may be prepared by synthesizing individual overlapping complementary oligonucleotides and filling in single stranded nonoverlapping portions using DNA polymerase in the presence of the deoxyribonucleotide triphosphates. This approach has been used successfully in the construction of several genes of known sequence. See, for example, Edge, M. D., Nature (1981) 292:756; Nambair, K. P., et al, Science (1984) 223:1299; Jay, Ernest, J Biol Chem (1984) 259:6311.

Synthetic oligonucleotides are prepared by either the phosphotriester method as described by Edge, et al, Nature (supra) and Duckworth, et al, Nucleic Acids Res (1981) 9:1691 or the phosphoramidite method as described by Beaucage, S.L., and Caruthers, M.H., Tet Letts (1981) 22:1859 and Matteucci, M.D., and Caruthers, M.H., J Am Chem Soc (1981) 103:3185 and can be prepared

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using commercially available automated oligonucleotide synthesizers. Kinasing of single strands prior to annealing or for labeling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 1 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 1-2 mM ATP, 1.7 pmoles  $\gamma$ 32P-ATP (2.9 mCi/mmole), 0.1 mM spermidine, 0.1 mM EDTA.

Once the components of the desired vectors are thus available, they can be excised and ligated using standard restriction and ligation procedures.

Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs, Product Catalog. In general, about 1  $\mu$ g of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20  $\mu$ l of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times of about one hour to two hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in Methods in Enzymology (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of E. coli DNA

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polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 min at 20 to 25°C in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM MgCl<sub>2</sub>, 6 mM DTT and 0.1-1.0 mM dNTPs. The Klenow fragment fills in at 5' single-stranded overhangs but chews back protruding 3' single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the overhang. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease or BAL-31 results in hydrolysis of any single-stranded portion.

Ligations are performed in 15-50 µl volumes under the following standard conditions and temperatures: for example, 20 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 33 µg/ml BSA, 10 mM-50 mM NaCl, and either 40 µM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 µg/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations are performed at 1 µM total ends concentration.

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIP) in order to remove the 5' phosphate and prevent self-ligation of the vector. Digestions are conducted at pH 8 in approximately 10 mM Tris-HCl, 1 mM EDTA using about 1 unit of BAP or CIP per µg of vector at 60° for about one hour. In order to

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recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion and separation of the unwanted fragments.

For portions of vectors derived from cDNA or genomic DNA which require sequence modifications, site specific primer directed mutagenesis may be used (Zoller, M.J., and Smith, M. Nucleic Acids Res (1982) 10:6487-6500 and Adelman, J.P., et al, DNA (1983) 2:183-193). This is conducted using a primer synthetic oligonucleotide complementary to a single stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the resulting partially or fully double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells which harbor the phage.

Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. The resulting plaques are washed after hybridization with kinased synthetic primer at a wash temperature which permits binding of an exact match, but at which the mismatches with the original strand are sufficient to prevent binding. Plaques which hybridize with the probe are then picked, cultured, and the DNA recovered.

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Verification of Construction

In the constructions set forth below, correct ligations for plasmid construction are confirmed by first transforming E. coli strain MC1061 obtained from Dr. M. Casadaban (Casadaban, M., et al, J Mol Biol (1980) 138:179-207) or other suitable host with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or using other markers depending on the mode of plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of Clewell, D.B., et al, Proc Natl Acad Sci (USA) (1969) 62:1159, optionally following chloramphenicol amplification (Clewell, D.B., J Bacteriol (1972) 110:667). Several mini DNA preps are commonly used, e.g., Holmes, D.S., et al, Anal Biochem (1981) 114:193-197 and Birnboim, H.C., et al, Nucleic Acids Res (1979) 7:1513-1523. The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy nucleotide method of Sanger, F., et al, Proc Natl Acad Sci (USA) (1977) 74:5463 as further described by Messing, et al, Nucleic Acids Res (1981) 9:309, or by the method of Maxam, et al, Methods in Enzymology (1980) 65:499.

The invention will be further described by the following examples. These are provided only to illustrate embodiments of the invention and are not to be construed as limitations on the invention's scope.

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Example 1Isolation of a Genomic Clone and cDNA Clones  
Encoding  $\beta$ -amyloid Core Protein  
and  $\beta$ -amyloid-related Proteins

5 A human genomic library in Charon 4A  $\lambda$ -phage was screened using a six-fold degenerate 38 mer probe corresponding to the first 13 amino acids of the 28 amino acid sequence N-terminal sequence. This probe,

10 3' CTGCGACTTAAGGCCGTGCTGAGICCGATGCTTCAGGTT-5'

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T

15 wherein I is inosine, when used to screen the human genomic library yielded a strongly hybridizing colony designated  $\lambda$ SM2.  $\lambda$ SM2 DNA was isolated and partially sequenced with the results shown in Figure 2. The sequenced portion is only a small fraction of the approximately 10-20 kb insert in the phage isolated from the genomic library.

20 A probe was constructed from the HindIII/RsaI fragment representing approximately positions 201-294. The genomic probe was used to screen a cDNA library prepared in  $\lambda$ gt10 using standard techniques from brain tissue of a 55 year old man with no evidence of Alzheimer's disease. The three clones designated  $\lambda$ SM2W4,  $\lambda$ SM2W3 and  $\lambda$ SM2W9 were identified.

Example 2

30 The genomic and cDNA sequences described above can be used to prepare recombinant protein in an efficient expression system. Genomic DNA can be utilized in cells, such as mammalian cells, capable of

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processing introns. Bacterial cells can be utilized for expression of cDNA sequences.

5 Bacterial Expression of  $\beta$ -Amyloid-Related Protein  
and Production of Antisera

A. Construction of plasmid pAPCP118-3.

Construction of an E. coli expression vector for human  $\beta$ -amyloid-related protein (655-751) required the joining of three DNA fragments: (1) a plasmid backbone (consisting of replication functions, ampicillin resistance gene, tryptophan promoter/operator, ribosome binding site, DNA encoding the amino terminus of E. coli  $\beta$ -galactosidase (7 amino acids) followed by six threonine residues, and transcription termination signals), (2) a fragment of the  $\beta$ -amyloid-related DNA encoding amino acids 655-728, of Figure 1 and (3) a synthetic fragment of the  $\beta$ -amyloid-related DNA encoding amino acids 729-751 of Figure 1 and the stop codon UAA.

The plasmid backbone referred to above is derived from pTRP83-1. Plasmid pTRP83-1 is a bacterial expression plasmid which was constructed in the following manner:

25

1. Construction of the Synthetic Tryptophan Operon Promoter and Operator Regulatory Sequence

The ten oligodeoxynucleotides shown in Figure 14 were synthesized by the phosphotriester method and purified. 500 pmole of each oligodeoxynucleotide except 1 and 10 were phosphorylated individually in 20  $\mu$ l containing 60 mM Tris-HCl, pH 8, 15 mM DTT, 10 mM MgCl<sub>2</sub>, 20  $\mu$ Ci of [ $\lambda$ -<sup>32</sup>P]-ATP and 20 units of polynucleotide kinase (P/L Biochemicals) for 30 min. at 37°C. This was followed by the addition of 10  $\mu$ l containing 60 mM

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Tris-HCl, pH 8, 15 mM DTT, 10 mM MgCl<sub>2</sub>, 1.5 mM ATP and 20 additional units of polynucleotide kinase followed by another 30 min incubation at 37°C. Following incubation the samples were incubated at 100°C for 5 min. 500  
5 pmole of oligodeoxynucleotides 1 and 10 were diluted to 30 µl in the above buffer without ATP.

16.7 pmole of each oligodeoxynucleotide constituting a double stranded pair (e.g. oligodeoxynucleotides 1 and 2, 3 and 4 etc. Figure 14 were  
10 mixed and incubated at 90°C for 2 min followed by slow cooling to room temperature. Each pair was then combined with the others in the construction and extracted with phenol/chloroform followed by ethanol precipitation. The oligodeoxynucleotide pairs were  
15 reconstituted in 30 µl containing 5 mM Tris-HCl, pH 8, 10 mM MgCl<sub>2</sub>, 20 mM DTT, heated to 50°C for 10 min and allowed to cool to room temperature followed by the addition of ATP to a final concentration of 0.5 mM. 800 units of T4 DNA ligase were then added and the  
20 mixture incubated at 12.5°C for 12-16 hours.

The ligation mixture was extracted with phenol/chloroform and the DNA ethanol precipitated. The dried DNA was reconstituted in 30 µl and digested with EcoRI and PstI for 1 hour at 37°C. The mixture was  
25 extracted with phenol/chloroform and ethanol precipitated followed by separation of the various double stranded DNA segments by electrophoresis on an 8% polyacrylamide gel, according to the method of Laemmli et al, Nature (1970) 227:680. The DNA fragments were  
30 visualized by wet gel autoradiography and a band corresponding to approximately 100 bp in length was cut out and eluted overnight as described. The excised synthetic DNA fragment was ligated to plasmids M13-mp8 or M13-mp9 (Messing and Vieira, (1982) Gene 19:259-268)  
35 similarly digested with EcoRI and PstI, and submitted to

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dideoxynucleotide sequence analysis to confirm the designed sequence. This designed sequence contains the promoter (-35 and -10 regions) and operator regions of the tryptophan operon (*trp*) as well as the ribosome binding region of the tryptophan operon leader peptide. Analogous sequences to that shown in Figure 14 have been proven to be useful in the expression of heterologous proteins in *E. coli* (Hallewell, R.A., and Entage, S., Gene (1980) 9:27-47, Ikehara, M., et al, Proc Natl Acad Sci (USA) (1984) 81:5956-5960).

## 2. Construction of the Synthetic *trp* Promoter/Operator Containing Plasmid pTRP233

Plasmid pKK233-2 (Amann, E. and Erosius, J., Gene (1985) 40:183 was digested to completion with *Nde*I and the ends were made blunt with 5 units of *E. coli* polymerase I, Klenow fragment (Boehringer-Mannheim, Inc.) and the addition of all four dNTPs to 50  $\mu$ M. This was incubated at 25°C for 20 min. Following phenol/chloroform extraction and ethanol precipitation, the *Nde*I-digested DNA was ligated and transformed into *E. coli* (Nakamura, K., et al, J Mol Appl Genet (1982) 1:289-299). The resulting plasmid lacking the *Nde*I site was designated pKK-233-2-Nde.

Twenty nanograms of plasmid pKK-233-2-Nde was digested to completion with *Eco*RI and *Pst*I followed by calf intestinal phosphatase treatment. Fifty nanograms of the synthetic *trp* promoter/operator sequence obtained from M13 RF, by digesting with *Eco*RI and *Pst*I, were mixed with ten nanograms of *Eco*RI and *Pst*I-digested pKK-233-2-Nde and ligated with T4-DNA ligase, followed by transformation into *E. coli* JA221 *lpp*<sup>-</sup>/*I*'*lac*I. Transformants were screened for the presence of plasmid DNA containing the 100 bp *Eco*RI-*Pst*I synthetic *trp*

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promoter/operator; the correct plasmid was then isolated and designated pTRP233.

pTRP233 was digested with *EccRI*, the ends blunted with Klenow, and ligated to remove the *EcoRI* restriction site. The plasmid was next digested with *NdeI* and *HindIII* and an *NdeI*-*EcoRI*-*HincIII* fragment encoding  $\beta$ -gal-(thr)6 between the *NdeI* and *EcoRI* sites was inserted to create plasmid pTRP83-1.

Plasmid pTRP83-1 was then digested with *EcoRI* and *HindIII* restriction endonucleases and the digest was electrophoresed in a 0.6% agarose gel (Maniatis, T. et al, at pp. 157-160). The large fragment containing the plasmid backbone was eluted from the gel. Next, the *EcoRI* fragment containing  $\beta$ -amyloid-related sequences derived from  $\lambda$ SM2W3 (corresponding to amino acids 655-751 of Figure 1 and 500 bp of 3'-untranslated sequences) was digested next with *HaeII* restriction endonuclease and electrophoresed in a 12% polyacrylamide gel. The approximately 230 bp *EcoRI*-*HaeII* fragment (containing  $\beta$ -amyloid-related sequences encoding amino acids 655-728) was eluted. The remaining portion of the  $\beta$ -amyloid-related sequences of Figure 1 encoding amino acids from 728-751 were prepared using the six oligodeoxynucleotides illustrated in Figure 9. 500 pmole of each oligodeoxynucleotide except for 1 and 6 were phosphorylated individually. 167 pmole of each oligodeoxynucleotide constituting a pair (e.g. 1 and 2, 2 and 3, etc.) were mixed and incubated at 90°C for 2 min followed by slow cooling to room temperature. Each pair was then combined with the others and extracted with phenol/chloroform followed by ethanol precipitation. The pairs were reconstituted in 30  $\mu$ l containing 5 mM Tris-HCl, pH 8, 10 mM MgCl<sub>2</sub>, 20 mM DTT, heated to 50°C for 10 min, and allowed to cool to room temperature. ATP was added to a final concentration of

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0.5 mM, 800 units of T4 DNA ligase was added and the mixture incubated at 12° C for 12-15 hr. The ligation was electrophoresed in a 12% polyacrylamide gel and the 79 bp HaeII-HindIII synthetic fragment was eluted.

5           The EcoRI-HindIII plasmic backbone of pTRP83-1, the approximately 230 bp EcoRI-HaeII  $\beta$ -amyloid cDNA fragment, and the 79 bp synthetic HaeII-HindIII  $\beta$ -amyloid fragment were ligated at 12°C for 12-16 hr. E. coli strain MC1061 was transformed with the ligation  
10 mixture (Maniatis, T. et al, pp. 250-251) and the resulting ampicillin resistant colonies were grown overnight in 1 ml of L broth supplemented with 100  $\mu$ g/ml ampicillin sulfate. Plasmid DNA was prepared by the alkaline lysis method (Maniatis et al, pp. 368-369).  
15 Plasmids were screened for the correct inserts by digestion with EcoRI and HindIII. A plasmid releasing an approximately 300 bp EcoRI-HindIII fragment was designated pAPCP118-3.

20   B. Expression of  $\beta$ -Amyloid-Related Fusion Polypeptide (655-751).

          The plasmid pAPCP118-3 expresses a 110 amino acid  $\beta$ -galactosidase-threonine- $\beta$ -amyloid-related fusion  
25 protein under the control of the E. coli tryptophan promoter/operator. E. coli strain W3110 was transformed with plasmid pAPCP118-3 and one of the resulting ampicillin resistant colonies was grown for 12-16 hr at 37°C in media containing M9 minimal salts (Miller, J., Experiments in Molecular Genetics, Cold Spring Harbor  
30 Laboratory, Cold Spring Harbor, New York) supplemented with glucose (0.4%), thiamine (2  $\mu$ g/ml),  $MgSO_4 \cdot 7H_2O$  (200  $\mu$ g/ml), tryptophan (40  $\mu$ g/ml), casamino acids (0.5%), and ampicillin (100  $\mu$ g/ml). Expression was induced by dilution of the culture 100-fold into new media with  
35 reduced tryptophan (4  $\mu$ g/ml) for 2 hr followed by the

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addition of 3- $\beta$ -indoleacrylic acid at a final concentration of 25  $\mu$ g/ml. Expression of  $\beta$ -gal-thr- $\beta$ -amyloid (655-751) fusion protein occurs at the level of 10-20% of total cell protein, and is present in the form of inclusion bodies which can be visualized by phase contrast microscopy (1000 x magnification). The cells were harvested 6 hr after the addition of the 3- $\beta$ -indoleacrylic acid by centrifugation, washed with 10 mM Tris-HCl, pH 7.5, and the cell pellet frozen at -20°C.

C. Purification of Beta-gal-thr- $\beta$ -Amyloid (655-751) Fusion Protein for Preparation of Antiserum.

A cell pellet from 500 ml of culture was resuspended in 40 ml of 10 mM Tris-HCl, pH 7.5, 0.6 M NaCl, and incubated with 8 mg of lysozyme and the protease inhibitors phenylmethylsulfonylfluoride (PMSF) and aprotinin (0.5 mM and 25  $\mu$ g/ml respectively) for 10 min at 4°C. Solutions of the two detergents, sodium deoxycholate (480  $\mu$ l of 10% solution) and NP-40 (240  $\mu$ l of 20% solution), were then added for an additional 10 min incubation at 4°C. The cell pellet was sonicated to disrupt cells and free inclusion bodies. RNase (10  $\mu$ g/ml) and DNase (10  $\mu$ g/ml) were added and the mixture stirred for 30 min at room temperature to digest RNA and DNA. The inclusion bodies (and some cell debris) were collected by centrifugation for 10 min at 5000 rpm (SA600 rotor). The supernatant was discarded and the pellet boiled in protein gel sample buffer for 20 min to solubilize the fusion protein. The fusion protein was then purified by electrophoresis in 12% SDS/polyacrylamide gels (Laemmli, U.K., Nature (1970) 227:680). The edges of each gel were removed and stained with Coomassie blue to visualize the 15 kilodalton (kD) fusion protein. They were then

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realigned with the gel so that the region of the gel containing the fusion protein could be excised. The polyacrylamide was then crushed through a series of needles (16 gauge down to 22 gauge) with the addition of physiological saline to keep the polyacrylamide moist. The polyacrylamide/fusion protein crush was mixed with adjuvant [RIBI(RAS)] just prior to immunization of the rabbits. Approximately 150-200 µg of fusion protein was administered per animal for the first immunization. Subsequent immunizations use 50-100 µg of fusion protein.

D. Western Blot Analysis of  $\beta$ -Amyloid Synpep Antisera Using Beta-gal-thr- $\beta$ -Amyloid (655-751) Fusion Protein.

Cell pellets of E. coli W3110 (pAPCP118-3) and W3110 (pTRP83-1) cultures induced with 3- $\beta$ -indoleacrylic acid were boiled in Laemmli gel sample buffer and electrophoresed in 12% SDS polyacrylamide. The second transformed strain is a negative control which contains all proteins except for the  $\beta$ -gal-thr- $\beta$ -amyloid (655-741) fusion. The gels were then electroblotted to nitrocellulose, incubated first with APCP synpep antisera collected from immunized rabbits, and then incubated with  $^{125}\text{I}$ -Staphylococcus protein A to identify bound antibody (Johnson, D.A., et al, Gene Anal Tech (1984) 1:3). An autoradiogram was generated from these nitrocellulose filters which demonstrated crossreactivity between anti-APCP3 serum and the fusion protein, Synpep APCP3 is comprised of amino acids 705-719 of Figure 1 which are included within the  $\beta$ -amyloid portion of the fusion protein. Cross-reactivity was also observed for other  $\beta$ -amyloid synpep antisera.

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Example 3

Generation of Polyclonal and Monoclonal Antibodies  
Against  $\beta$ -Amyloid-Related Protein Using Live  
Recombinant Vaccinia Virus

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1. Construction of Plasmid pFL4T4B.

The construction of the plasmid which allowed for the generation of polyclonal and monoclonal antibodies is schematically represented in Figure 10. Plasmid pGEM-3<sup>TM</sup> (Promega-Biotec) was EcoRI-digested and treated with calf intestinal phosphatase in accordance with Maniatis, et al. Fifty nanograms of the purified 1.06 kb EcoRI fragment derived from  $\lambda$ APCP168i4 were mixed with 10 nanograms EcoRI digested pGEM-3<sup>TM</sup> and incubated with T4 DNA ligase in a total volume of 20  $\mu$ l for 30 min at 25°C. *E. coli* strain MC1061 was made competent for transformation by the CaCl<sub>2</sub> method and transformed with the ligation mix. Resulting ampicillin resistant colonies were grown overnight in 2 ml L-amp broth from which plasmid DNA was prepared by the Triton lysis method (Maniatis et al). Plasmids were screened for the correct orientation by digestion with HindIII. A plasmid having 150 and 3700 bp HindIII restriction fragments was chosen and designated p4BI. The resulting plasmid p4BI was digested with HindIII, religated with T4 ligase for 30 minutes at 25°C and competent MC1061 cells were transformed with the ligation mixture. Plasmids were screened for loss of the 130 bp HindIII fragment by EcoRI digestion. A plasmid containing a single EcoRI site was chosen and designated p4BARI. Ten nanograms of plasmid p4BARI was EcoRI-digested, treated with calf intestinal alkaline phosphatase, and ligated with 100 nanograms of the purified ~2 kb EcoRI fragment derived from  $\lambda$ APCP168i4. The ligation mixture was used

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to transform competent MC1061 cells. Resulting  
ampicillin-resistant colonies were grown overnight in  
L-amp broth and plasmid DNA was prepared. Plasmids were  
5 screened for the correct orientation by digestion with  
BamHI and HindIII. A plasmid having a 1.5 kb BamHI and  
an ~1.5 kb BamHI-HindIII fragment was chosen and  
designated p4T4B. Plasmid p4T4B was digested with SmaI  
and XmnI and the resulting ~2.7kb fragment was eluted  
10 from 0.8% agarose followed by ethanol precipitation,  
dried in vacuo and resuspended in dH<sub>2</sub>O.

Five µg of the vaccinia virus expression  
vector pSC11 (Chakrabarti, et al, Mol Cell Biol (1985)  
5:3403-3409) were digested to completion with SmaI  
followed by treatment with calf intestinal phosphatase.  
15 Five hundred nanograms of the purified ~2.7 kb SmaI-XmnI  
fragment derived from p4T4B were mixed with fifty  
nanograms of SmaI-digested pSC11 and incubated with T4  
DNA ligase in a total volume of 20 µl for 16 hours at  
15°C overnight. E. coli strain MC1061 was transformed  
20 with the ligation mix. Resulting ampicillin resistant  
colonies were grown overnight and plasmid DNA was  
isolated by the rapid boiling method (Maniatis et al).  
Plasmids were screened for insertion and correct  
orientation by digestion with EcoRI. A plasmid having  
25 both an ~2500 bp and an ~600 bp EcoRI fragment was  
chosen and designated pFL4T4BV.

Monoclonal and polyclonal antibodies against  
full length β-amyloid-related protein are generated by  
using a novel method described by Yilma, T., et al,  
30 (Hybridoma (1987) 6:329-337). Briefly, the method  
enables the production of antibodies to a specified  
protein without the need for a purified antigen  
(protein) in either the immunization or screening phase  
of the procedure. The methods make use of the vaccinia  
35 virus cloning vectors (Smith, et al, Nature (1983)

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302:490-495) which can be genetically engineered to carry isolated genes. The infectious recombinant vaccinia virus may then be used to immunize mice. Two weeks after infection, mice are sacrificed and their spleen cells are fused with myeloma cells for monoclonal antibody production as described in the classical approach developed by Kohler and Milstein Nature (1973) 256:495. Alternatively, rabbits can be conventionally immunized with the infectious vaccinia virus recombinant to generate polyclonal antisera.

Ten  $\mu$ g of plasmid pT4BV is used to transfect CV-1 monkey kidney cells infected with wild-type vaccinia virus according to standard methods (Mackett, et al, J Virol (1984) 49:857-864). TK<sup>-</sup> recombinants are isolated by plaque assay on TK<sup>-</sup> cells in the presence of 25  $\mu$ g/ml Bromodeoxyuridine (BUDR). For plaque assays involving blue color production, as in the case of the pSC11 vaccinia virus coexpression vector, 300  $\mu$ g of X-Gal per milliliter is placed in the agarose overlay, and plaques visualized after 4-6 hrs at 37°C. Plaques are purified two to three times in succession. DNA from the recombinant virus is examined by restriction endonuclease analysis and DNA hybridization to <sup>32</sup>P-nick-translated 2091 bp EcoRI fragment from  $\lambda$ APCP168i4 to confirm the predicted structure.

Recombinant virus carrying the complete  $\beta$ -amyloid-related cDNA sequence of  $\lambda$ APCP168i4 is isolated and amplified to high titer ( $1 \times 10^{8-9}$  pfu/ml). These recombinant viruses are used to immunize rabbits and mice for the subsequent production of polyclonal and monoclonal antibodies respectively, against full length  $\beta$ -amyloid-related protein(s) using well established methods or they can be used for the direct expression of the recombinant protein. The various antisera are screened either for their ability to specifically

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immunoprecipitate the correct size protein from  
35S-methionine-labeled CV-1 cells which have been  
infected with an  $\beta$ -amyloid-related protein virus  
recombinant or for their ability to detect denatured  
5 protein on a western blot of similar cells which have  
not been exposed to radiolabeled amino acid.

#### Example 4

##### 10 Expression of $\beta$ -Amyloid-Related Protein (1-751) in Cultured Mammalian Cells

To facilitate the expression of  
 $\beta$ -amyloid-related protein in mammalian cells, a plasmid  
is constructed such that the coding segment for the  
15 protein is fused to a powerful regulated promoter  
derived from the human metallothionein II (hMTII) gene.  
This procedure is performed in two steps. First an  
expression vector pMTSV40 polyA Bam was derived from  
phGH-SV(10) vector by digestion of phGH-SV(10) with  
20 BamHI and SmaI restriction enzymes, followed by  
incubation with DNA polymerase I (Klenow fragment) in  
order to create blunt-ended molecules. The blunt ends  
are subsequently ligated to BamHI linkers, cut with  
BamHI, and religated to allow for recircularization.  
25 This step removes all of the human growth hormone  
genomic sequence from phGH-SV(10) except for most of the  
3' untranslated region of the mRNA and genomic sequences  
encoding putative 3' transcriptional stop and processing  
signals. For the mammalian cell expression construct,  
30 pMTSV40 polyA Bam is BamHI-digested, then incubated with  
all four nucleotide triphosphates and with DNA  
polymerase I to create blunt ends. This fragment is  
subsequently ligated with the purified 2678 bp SmaI-XmnI  
fragment derived from p4T4B (described previously). The

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recombinant molecules are introduced into MC1061 by transformation.

Chinese hamster ovary (CHO)-K1 cells are grown in a medium composed of a 1:1 mixture of F12 medium and DME medium with 10% fetal calf serum. The competent cells are co-transformed with the recombinant expression vector and pSU2:NEO (Southern, P., et al, J Mol Appl Genet (1982) 1:327-341). pSV2:NEO contains a functional gene conferring resistance to the neomycin analog G418. In the transformation, 500 ng of pSV2:NEO and 5 µg of the recombinant vector are applied to a 60 mm dish of CHO cells as a calcium phosphate-DNA co-precipitate as described by Graham, F.L. and Van der Eb, A.J. Virology (1973, 52:456-467. Growth of the cells in the antibiotic G418 as described by Southern et al will yield a pool of stably transfected CHO cells containing expression vector DNA with the capacity to express β-amyloid-related mRNA and protein.

#### Example 5

##### Expression of β-Amyloid-Related Protein (652-751) in Cultured Mammalian Cells

A mammalian cell expression vector encoding for the production of a β-amyloid-related protein can be constructed as shown in Figure 12 as follows: the p4BARI vector of Figure 10 is linearized by digestion with EcoRI. The vector is mixed with two oligonucleotides having the sequences:

5'-ATTCCCGGGACCATGGATGCAG-3'

3'-GGCCCTGGTACCTACGTCTTAA-5'

and ligated using T4 DNA ligase. These oligonucleotides reconstruct the Met-Asp-Ala codons of λSM2W4 and precede

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them by EcoRI and SmaI sites and follow them with another EcoRI site.

Competent E. coli strain DH1 cells are transformed with the mixture and ampicillin-resistant bacteria are selected by growth on L-Amp plates. A transformant containing the oligonucleotide pair inserted into the EcoRI site in the proper orientation is selected by standard screening techniques and designated pΔW4/W3. Plasmid DNA pΔW4/W3 is digested with SmaI and XmnI to remove sequences encoding the β-amyloid-related protein described in Figure 5 and the correct piece is isolated by gel purification.

This piece can then be inserted into the mammalian cell expression vector pMTSV40 polyA Bam which has been linearized with BamHI and rendered blunt-ended as described above in Example 4. The resulting vector, PMT-APCP (652-751) can be used for the production of the β-amyloid-related protein (652-751).

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#### Example 6

##### Expression of β-Amyloid Precursor in Mammalian Cells

Outlined in Examples 4 and 5 are the construction of an expression system for the β-amyloid-related protein (1-751) driven by the human β-actin promoter. A nearly identical construct was prepared using the purified 2548 bp SmaI-XmnI fragment derived from p4T4B (described previously in Example 3) from which 116 bp from the 5' untranslated region have been deleted. This fragment was inserted into the SalI site behind the human β-actin promoter on a plasmid harboring the neomycin selectable marker for mammalian cell expression and the ampicillin resistance gene for selection of bacterial transformants. This vector, pHbAPr-1-neo, has been described by Gunning, et al,

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(Proc Nat'l Acad Sci USA (1987) 84:4831-4835) and has been modified to remove the EcoRI site from the body of the original vector and to substitute the original polylinker region with a new polylinker containing an EcoRI site in addition to the SalI, HindIII, and BamHI cloning sites originally present. The modified vector is referred to as pAXneoR. The pAXneoR vector was linearized with SalI, the termini filled in using Klenow fragment of DNA polymerase to create blunt-ended molecules. The 2548 bp SmaI-XmI  $\beta$ -amyloid fragment was blunt-ligated into the vector using T4 ligase. The recombinant molecules were introduced into E. coli MC1061 by transformation and a clone displaying the proper orientation was amplified. A similar construction was made using the 695  $\beta$ -amyloid sequences described by Kang et al (supra) which places the 695 amyloid protein under control of the human  $\beta$ -actin promoter.

600  $\mu$ g total DNA of pAXneo/751  $\beta$ -amyloid or pAXneo/695  $\beta$ -amyloid or an equal mass mixture of both plasmid constructs were introduced into  $10^7$  CHO cells by electroporation (Neumann, J Membrane Biol (1972) 10:279-290; Zimmerman, Biophys J (1973) 13:1005-1013) using a BTX Transfector 100, Bio-Rad sterile, disposal cuvettes and a custom built cuvette holder. G418-resistant cells receiving the exogenous DNA were selected by standard protocols (Southern, 1982, supra) using 500  $\mu$ g/ml G418 from Gibco.

The pool of positively transfected cells resistant to G418 from each of the three transfections was characterized with respect to  $\beta$ -amyloid precursor protein expression. Approximately  $2 \times 10^6$  cells from each pool containing 5 mls of serum-free medium were incubated at 37°C for 48 hrs. The conditioned media was removed and the protein precipitated by addition of

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trichloroacetic acid to a final concentration of 10%. Cells were harvested by scraping, washed in saline buffered with phosphate and resuspended in 50 ul of buffer for a 30-fold concentration. 25 ul of each sample was loaded onto a 12.5% polyacrylamide gel (Laemmli, Nature (1970) 277:680-685). The  $\beta$ -amyloid precursor was detected by Western blot analysis (Towbin, Proc Nat'l Acad Sci USA (1979) 76:4350-4354) using standard procedures and  $\beta$ -amyloid-specific polyclonal antibodies generated by recombinant vaccinia virus harboring the  $\beta$ -amyloid 751 cDNA as described in Example 3. Typically, the majority of the approximately 110,000 dalton  $\beta$ -amyloid precursor is found to be released into the culture media and very small amounts of the protein is cell-associated. This result is in keeping with the hypothesis of Allsop, et al, (Proc Natl Acad Sci USA (1988) 85:2790-2794) proposing that the  $\beta$ -amyloid protein is a secreted prohormone. The apparent molecular weight of 110,000 daltons of the recombinantly expressed  $\beta$ -amyloid protein is similar to that observed by others (Dyrks, T., et al, EMBO J (1988) 7(4):949-957) using in vitro transcription/translation systems.

The  $\beta$ -amyloid 751 protein cloned into a vaccinia virus as described in Example 3 was also examined for the nature of  $\beta$ -amyloid protein expression. The purified recombinant virus was used to infect  $10^6$  CV-1 cells at a MOI of 1 under serum-free conditions. 18 hrs post infection with the virus, both cells and supernatants were harvested, subjected to polyacrylamide gel electrophoresis and Western blotting using the polyclonal antisera described above. As shown in Figure 15, the  $\beta$ -amyloid 110,000 dalton protein was found to be present in the conditioned media versus associated with the cell.

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Example 7Assay to Distinguish Genetic Variants  
of B-Amyloid-Related Protein mRNA Species

5           The ability to distinguish between genetic variants of  $\beta$ -amyloid-related protein mRNA species using oligonucleotide probes is demonstrated herein.

          A diagnostic assay for Alzheimer's disease might take the form of distinguishing between two  
10       closely related genetic variants of  $\beta$ -amyloid-related proteins or their mRNAs, and quantitating the relative levels of expression of these proteins or mRNAs. Figure 8 provides an example of the use of the invention sequences to provide a standard for the diagnostic  
15       assay.

          Total cellular RNA or cytoplasmic RNA was prepared from human cells in culture or human brain tissue (Alzheimer's brain or normal brain) with or without removal of nuclei (cytoplasmic or total,  
20       respectively) by the guanidine thiocyanate/CsCl method as described by Maniatis et al. The samples corresponding to the numbering in Figure 8 are: (1) total RNA from IMR-32 cells (ATCC #CCL127), a mixed neuroblastoma and fibroblast culture; (2) total RNA from  
25       MRC5 cells (ATCC #CCL171), a normal fibroblast; (3) total RNA from HeLa cells (ATCC #CCL2.2), an epitheloid cell; (4) cytoplasmic RNA from MRC5 cells; (5) cytoplasmic RNA from HeLa cells; (6) total RNA from HL-60 cells (ATCC #CCL240), a promyelocytic leukemia;  
30       (7) total RNA from HL-60 cells which have been treated with 12-tetra-decanoyl-phorbol-13-acetate to induce differentiation of the cells to macrophages; (8) total RNA from normal cerebellum samples; (9) total RNA from normal frontal cortex samples; (10) total RNA from an  
35       Alzheimer's individual's frontal cortex; and (11) total



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RNA from a normal parietal cortex. RNA was fractionated by oligo-dT cellulose chromatography, electrophoresed on a formaldehyde agarose gel, and blot-transferred to nitrocellulose (all as described in Maniatis et al).

5 Filters were baked, prehybridized and hybridized to the indicated probes according to standard protocols.

The probes indicated are: (1) Junction, a 30 base oligonucleotide #2733, specific for the Kang et al sequence, as described above in the detailed description of the invention; (2) Insert, a 60 base oligonucleotide #2734 specific for the  $\beta$ -amyloid-related sequences described in Figure 1, and as described above; and (3) an 1800 bp human actin cDNA insert, isolated from the plasmid pHFBA-1 (Ponte, P., et al, Nuc Acids Res (1984) 12:1687-1696. Oligonucleotide probes were end-labeled with [ $^{32}$ P]-dCTP by incubation with terminal transferase according to manufacturer's suggestions. Actin insert was radiolabeled with [ $^{32}$ P]-CTP by nick-translation. After hybridization, the filters hybridized to oligonucleotides were washed at 1 x S.S.C., 55° C. The filter hybridized to actin was washed at 0.1 x SSC at 55°C. Filters were then exposed to X-ray film to produce the autoradiogram shown. The insert probe detects the  $\beta$ -amyloid related protein mRNA described in Figure 1 in all samples examined. The junction probe detects the  $\beta$ -amyloid-related mRNA described by Kang et al in all cells except HeLa and MRC5. The actin probe is a control which is expected to hybridize to an abundant RNA in all cells.

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Example 8Bacterial Expression of  $\beta$ -Amyloid-Related Protein (289-345)

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A. Construction of Plasmid pAPCP125-2.

A synthetic gene was assembled according to the teaching of Example 2 for  $\beta$ -amyloid-related protein (289-345) from three pairs of oligodeoxyribonucleotides (illustrated in Figure 9D) utilizing E. coli preferred codon choice for highly expressed genes, and a hydroxylamine cleavage site (Asn-Gly) was inserted preceding amino acid 289 (Glu) to permit release of the polypeptide from a fusion protein. The expression vector pTRP83-1 was digested with restriction endonucleases EcoRI and HindIII and the linearized plasmid purified from a 0.6% agarose gel. Fifty  $\mu$ g of plasmid DNA and 200  $\mu$ g of synthetic gene DNA were ligated using T4 DNA ligase and E. coli MC1061 was transformed with the ligation. Ampicillin-resistant colonies were grown overnight in L broth containing 100  $\mu$ g/ml ampicillin and alkaline plasmid preps were made. The resulting plasmid DNA was digested with BamHI restriction endonuclease to confirm insertion of the gene within the vector by release of an approximately 350 bp fragment. One plasmid receiving the synthetic gene insert was designated pAPCP125-2.

B. Expression of  $\beta$ -Amyloid-Related Fusion Polypeptide (289-345).

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The plasmid pAPCP125-2 is designed to express a 74 amino acid  $\beta$ -galactosidase-threonine- $\beta$ -amyloid-related fusion protein under the control of the E. coli tryptophan promoter/operator. E. coli strain W3110 is transformed

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with plasmid pAPCP125-2 and one of the resulting  
ampicillin resistant colonies is grown as described in  
Example 2. Expression is induced by the addition of  
3- $\beta$ -indoleacrylic acid at a final concentration of 25  
5  $\mu$ g/ml. After 5 hrs induction, a 1 ml aliquot of cells  
is withdrawn from the culture, harvested by  
centrifugation, then boiled in 100  $\mu$ l of Laemmli protein  
sample buffer for electrophoresis through a 16%  
SDS-polyacrylamide gel by standard methodologies.  
10 Assessment of inclusion body formation is made by phase  
contrast microscopy (1000X). Expression levels are  
estimated by Coomassie blue staining of the gel followed  
by densitometer scan to quantitate the intensity of  
protein bands. Cells to be used for protein  
15 purification are harvested by centrifugation, washed  
with 10 mM Tris-HCl, pH 7.5, and the cell pellet frozen  
at -20°C until needed.

C. Purification of Beta-gal-thr- $\beta$ -amyloid-related  
20 Protein (289-345)

The fusion protein is purified as described  
for the  $\beta$ -gal-thr- $\beta$ -amyloid-related (655-751) fusion  
protein (Example 2) in the absence of PMSF and  
aprotinin. A series of washes from 2 M urea to 4 M urea  
25 removes other proteins and further enriches fusion  
protein found in inclusion bodies. If further  
purification is desired, the fusion protein is  
solubilized in 6-8 M urea, and a gel filtration or ion  
exchange chromatography step is included. If not, the  
30 fusion protein is solubilized in 6 M guanidium  
hydrochloride with hydroxylamine under the conditions  
described by Moks, et al., Biochem (1987) 26:5239-5244  
for cleavage between the Asn and Gly residues releasing  
 $\beta$ -amyloid-related protein (289-345) with a Gly residue  
35 at its amino-terminus. The cleaved peptides are

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purified by reversed phase high pressure liquid chromatography, ion exchange or gel filtration chromatography. The purified  $\beta$ -amyloid-related protein is then reduced and reoxidized by methods described by Tan and Kaiser, J Org Chem (1976) 41:2787 and Biochemistry (1977) 16:1531-1541, to reform disulfide bonds between the six Cys residues. Successful reoxidation of bovine pancreatic trypsin inhibitor (aprotinin) also containing six Cys residues and produced in E. coli has been accomplished by these methods (von Wilcken-Bergmann, et al, EMBO (1986) 5:3219-3225).

#### Example 9

##### Construction and Expression of the Inhibitor Protein

DNA sequences coding for each of the two chimeric proteins were assembled from synthetic oligonucleotides. The sequences of the oligonucleotides used are shown in Figure 16. The sequence of the phoA signal peptide (Figure 16B) is from Kikuchi et al. (supra), the sequence for ompA signal peptide (Figure 16A) is from Beck and Bremer, (supra). Each oligonucleotide was treated with kinase (except for the 2 outside 5' ends).

All 8 oligonucleotides encoding either the phoA or ompA fusions were mixed together and treated with ligase. Analytical gels showed a new band of the expected length (~250 bp). The ligated constructs were then ligated into the NdeI-HindIII sites of the vector pTRP233. The ligated vectors were transfected into E. coli strain MC1061 and Amp<sup>R</sup> colonies selected. Plasmid minipreps showed recombinant plasmids with the correct restriction map. Miniprep DNA was used to transfect strains W3110 and JE5505. Small scale cultures of each

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of the three strains were grown and induced with IAA overnight. Culture supernatants were examined for trypsin inhibitory activity. Trypsin is assayed for its ability to hydrolyze the synthetic substrate  
5 N-benzoyl-D-arginine-p-nitroaniline to release p-nitroaniline (pNA). The release of pNA as a function of time is easily monitored in a spectrophotometer and can be quantitated to measure trypsin activity. The inhibitor is detected in this assay by virtue of its  
10 ability to bind to trypsin and prevent hydrolysis of the substrate by trypsin. Inhibitory activity was detected in the culture medium for both ompA and phoA constructs in JE5505 but not W3110 or MC1061. Expression levels appeared to be higher with the phoA construct and so  
15 only this construct was used for subsequent experiments.

A time course study was conducted in which levels of inhibitor in the medium were assayed and rates of synthesis of the inhibitor were monitored by <sup>35</sup>S-methionine incorporation into inhibitor protein.  
20 This study showed that synthesis declined to zero between 4 and 6 hrs after induction with IAA while inhibitor protein accumulated in the medium out to 8 hrs post-induction. This lag is presumed to represent the time required for protein to diffuse from the periplasm  
25 through the outer membrane into the medium. Levels of inhibitor in the medium appeared to remain stable from 8 to 24 hrs post-induction.

#### Example 10

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##### Purification of Inhibitor Protein

A 5 liter culture of E. coli JE5505 transformed with the phoA construct was grown overnight, induced at OD<sub>550</sub>=0.1, and harvested at 8 hrs after  
35 induction with IAA. Cells were centrifuged out and

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discarded. The supernatant was filtered through 8  $\mu$ m and 0.45  $\mu$ m filters and passed through a trypsin Sepharose affinity column (total 10 ml Sepharose, 6 mg/ml trypsin on Sepharose, 5 ml/min flow rate, 4°C).

5 The column was washed with 0.1 M sodium acetate buffer, pH 4, containing 0.3 M sodium chloride (NaCl) and 0.01 M calcium chloride (CaCl<sub>2</sub>) to remove nonspecifically bound protein. The inhibitor was eluted with a buffer of 0.1 M hydrochloric acid-0.5 M NaCl-0.01 M CaCl<sub>2</sub>, pH 1.25.

10 Alternatively, rather than using trypsin affinity column as the affinity matrix, a trypsin bead slurry may be employed. To 5 liters of the E. coli JE5505 supernatant, about 20 mls of a trypsin Sepharose bead slurry were added and stirred gently with a mixer (at

15 300 rpm, 1 hr, room temperature). The mixture was decanted into a scintered glass funnel and the liquid aspirated from the beads. Using approximately 4 liters of 20 mM Tris-HCl, pH 7.5, the beads were re-equilibrated and then washed with 0.1 M acetic

20 acid-0.3 M NaCl, pH 4.5. The beads were re-equilibrated using 20 mM Tris-HCl, pH 7.5 and then the protease inhibitor was eluted using about 80 mls of 0.1 M HCl-0.5 M NaCl, pH 1.25. The eluate was neutralized using approximately 2.5 mls of 2 M Tris base, pH 10.0.

25 The trypsin affinity column eluate was injected onto a Jones Chromatography APEX-WP<sup>®</sup> butyl HPLC column (1 cm ID x 25 cm length) equilibrated in 20% acetonitrile-0.1% trifluoroacetic acid-80% water. A linear gradient to 60% acetonitrile/0.1% TFA in H<sub>2</sub>O was

30 run to eluate the inhibitor. The inhibitor elutes in a major peak (peak 4) and a minor peak (peak 2). Both are active in the trypsin inhibition assay, both appear homogeneous on the protein sequencer (40 cycles for peak 4, 49 cycles for peak 2) and both have the amino acid

35 composition expected for the A4 inhibitor. Treatment of

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peak 2 with 10 mM DTT (dithiothreitol) causes partial conversion of peak 2 to peak 4, suggesting that peak 2 may rise by oxidation of methionine. In each case the endogenous *E. coli* signal peptidase had cleaved the chimeric protein at the expected site as shown by the arrows in Figure 16. Mass spectrometric (MS) analysis indicates that peak 4 has a molecular mass of 6,267 daltons, very close to the predicted value of 6,267.7 for full length A4<sub>i</sub> with 3 disulfide bridges. Since each S-S bridge formed results in loss of 2 H<sup>+</sup> (= 2 daltons), the number of S-S can be assessed. Peak 2 gives a heterogeneous peak in MS about 80 daltons greater than peak 4, consistent with oxidation. The acid conditions used to elute the protein from the trypsin-Sepharose affinity column will promote oxidation of methionine, however, peak 2 formation is minimized by rapid neutralization using a buffered solution, such as, for example, 2 M Tris base having a pH in the range of about 8 to about 11, preferably pH 10.0, after elution from the trypsin-Sepharose affinity column.

While preferred embodiments of making and using the invention have been described, it will be appreciated that various changes and modifications can be made without departing from the invention.

The following cultures have been deposited with the American Type Culture Collection (ATCC), Rockville, MD, USA for patent purposes. Bacteriophage phages λSM2, λSM2W9, and λAPCP168i4 were deposited under the conditions specified by the Budapest Treaty on the International Recognition of the Deposit of Microorganisms (Budapest Treaty).

	<u>Culture</u>	<u>Accession No.</u>	<u>Deposit Date</u>
	λSM2	40279	13 November 1986
35	SM2W4	40299	29 December 1986

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SM2W3	40300	29 December 1986
λSM2W9	40304	29 January 1987
λACPC168i4	40347	1 July 1987

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Availability of the deposited strains are not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

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Claims

- 5           1. An isolated native, cloned recombinant or synthetic DNA sequence useful in the prognosis and diagnosis of Alzheimer's disease in human subjects comprising the DNA sequence of Figure 1.
- 10           2. A subfragment of the DNA sequence of claim 1 wherein the subfragment corresponds to the 168 basepair insert fragment of the  $\beta$ -amyloid-related gene product of bacteriophage  $\lambda$ APCP168i4.
- 15           3. An isolated native, cloned recombinant or synthetic DNA sequence corresponding to nucleotides 864 to 1035 shown in Figure 1.
- 20           4. The DNA of claim 2 wherein the  $\beta$ -amyloid-related protein has the amino acid sequence:
- 25           GluValCysSerGluGlnAlaGluThrGlyProCysArgAlaMet  
            IleSerArgTrpTyrPheAspValThrGluGlyLysCysAla  
            ProPhePheTyrGlyGlyCysGlyGlyAsnArgAsnAsnPhe  
            AspThrGluGluTyrCysMetAlaValCysGlySerAlaIle.
- 30           5. Recombinant  $\beta$ -amyloid-related protein obtained by the expression of the DNA of claim 1.
6. Recombinant  $\beta$ -amyloid-related protein obtained by the expression of the DNA of claim 2.
7. Recombinant  $\beta$ -amyloid-related protein obtained by the expression of the DNA of claim 3.
- 35           8. The cloning vector  $\lambda$ APCP168i4.

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9. A protease inhibitor having the amino acid sequence:

5           GluValCysSerGluGlnAlaGluThrGlyProCysArgAlaMet  
          IleSerArgTrpTyrPheAspValThrGluGlyLysCysAla  
          ProPhePheTyrGlyGlyCysGlyGlyAsnArgAsnAsnPhe  
          AspThrGluGluTyrCysMetAlaValCysGlySerAlaIle.

10           10. A biologically active, refolded and  
          substantially purified protease inhibitor having the  
          amino acid sequence:

          GluValCysSerGluGlnAlaGluThrGlyProCysArgAlaMet  
15           IleSerArgTrpTyrPheAspValThrGluGlyLysCysAla  
          ProPhePheTyrGlyGlyCysGlyGlyAsnArgAsnAsnPhe  
          AspThrGluGluTyrCysMetAlaValCysGlySerAlaIle.

          11. An analog of human amyloid plaque core  
20           protein protease inhibitor wherein the amino acid  
          corresponding to arginine at position 13 in the sequence

          GluValCysSerGluGlnAlaGluThrGlyProCysArgAlaMet  
          IleSerArgTrpTyrPheAspValThrGluGlyLysCysAla  
25           ProPhePheTyrGlyGlyCysGlyGlyAsnArgAsnAsnPhe  
          AspThrGluGluTyrCysMetAlaValCysGlySerAlaIle

          is substituted with an aromatic amino acid, said analog  
          exhibiting chymotrypsin inhibitory activity.

30

          12. The analog of claim 11 wherein said  
          aromatic amino acid is selected from the group  
          consisting of phenylalanine, tyrosine and tryptophan.

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13. An analog of human amyloid plaque core protein protease inhibitor wherein the amino acid corresponding to arginine at position 13 in the sequence:

5

GluValCysSerGluGlnAlaGluThrGlyProCysArgAlaMet  
IleSerArgTrpTyrPheAspValThrGluGlyLysCysAla  
ProPhePheTyrGlyGlyCysGlyGlyAsnArgAsnAsnPhe  
AspThrGluGluTyrCysMetAlaValCysGlySerAlaIle

10

is substituted with neutral hydrophobic amino acid, said analog exhibiting human elastase inhibitory activity.

15

14. The analog of claim 13 wherein said neutral hydrophobic amino acid is selected from the group consisting of leucine, methionine and valine.

20

15. A method for treating acute pancreatitis in a subject which method comprises administering to said subject an effective amount of the protease inhibitor of claim 9.

25

16. A pharmaceutical composition comprising the protease inhibitor of claim 9 in admixture with a pharmaceutically acceptable excipient.

30

17. A method of diagnosing a genetic predisposition to Alzheimer's disease in a test subject, comprising:

identifying, as being associated with predisposition to Alzheimer's disease, one or more alterations in the DNA of claim 1; and

assaying test subject gene fragments for the presence or absence of such alteration(s).

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18. The method of claim 17 wherein the alteration effects the 168 basepair insert of  $\lambda$ APCP168i4.

5 19. A method of diagnosing Alzheimer's disease in a test subject, comprising:

preparing a peptide which includes an immunogenic region of the protein of claim 5;  
eliciting antibodies which are specific  
10 against said peptide; and

using the antibodies to detect an increase or decrease of  $\beta$ -amyloid-related proteins in a test subject suspected of having Alzheimer's disease.

15 20. A method of diagnosing Alzheimer's disease in a test subject, comprising:

preparing a peptide which includes an immunogenic region of the protein of claim 6;  
eliciting antibodies which are specific  
20 against said peptide; and

using the antibodies to detect the increase or decrease of  $\beta$ -amyloid-related proteins in a test subject suspected of having Alzheimer's disease.

25 21. The method of claim 20 wherein said antibodies are monoclonal.

30 22. A reagent for use in the diagnosis of Alzheimer's disease in a human patient comprising the DNA sequence of claim 2.

23. The diagnostic reagent of claim 19 which is labelled.

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24. A method for producing the protease inhibitor of claim 9 from a transformed microorganism as a secreted, properly folded protein, which method comprises:

5 a) constructing a chimeric DNA sequence encoding a signal peptide sequence fused in phase with the protease inhibitor sequence;

10 b) expressing the chimeric DNA sequence in a bacterial host strain capable of secreting the protein into the culture medium, which host has been transformed with a vector capable of expressing the chimeric DNA sequence; and

15 c) recovering the secreted, properly folded protein.

25. The method of claim 24 wherein the DNA sequence encoding the signal peptide sequence is selected from the group consisting of the ompA or phoA secretion signal sequences.

20

26. The method of claim 24 wherein the bacterial host cell is E. coli JE5505.

25 27. A method for recovering the refolded, substantially purified protease inhibitor of claim 10 from a transformed microorganism containing a DNA sequence encoding the protease inhibitor, which method comprises:

30 a) harvesting the bacterial broth from the transformed, cultured microorganism;

b) separating the soluble material containing the protease inhibitor from the bacterial cell;

35 c) reacting the soluble material with an affinity matrix to which a serine protease is bound to capture the protease inhibitor;

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d) eluting the captured p-otease inhibitor from said matrix under acidic conditions; and

e) purifying the protease inhibitor in the eluate by high performance liquid chromatography to recover the refolded, substantially purified protease inhibitor.

28. The process of claim 27 wherein the serine protease is trypsin or chymotrypsin.

29. The process of claim 27 wherein the acidic conditions employ lowering the pH to a range of about 1 to about 5.

30. The process of claim 29 wherein the pH is 1.25.

31. The process of claim 29 wherein the acid elution step is followed by rapid neutralization using a buffered solution having a pH in the range of about 8 to about 11.

32. The process of claim 31 wherein the buffered solution is 2 M Tris base having a pH of 10.

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																		ATG	CTG	CCC
																		MET	Leu	Pro
GGT	TTG	GCA	CTG	CTC	CTG	CTG	GCC	GCC	TGG	ACG	GCT	CGG	GCG	CTG	GAG	GTA	CCC			
Gly	Leu	Ala	Leu	Leu	Leu	Leu	Ala	Ala	Trp	Thr	Ala	Arg	Ala	Leu	Glu	Val	Pro			
										10									20	
ACT	GAT	GGT	AAT	GCT	GGC	CTG	CTG	GCT	GAA	CCC	CAG	ATT	GCC	ATG	TTC	TGT	GGC			
Thr	Asp	Gly	Asn	Ala	Gly	Leu	Leu	Ala	Glu	Pro	Gln	Ile	Ala	MET	Phe	Cys	Gly			
										30										
AGA	CTG	AAC	ATG	CAC	ATG	AAT	GTC	CAG	AAT	GGG	AAG	TGG	GAT	TCA	GAT	CCA	TCA			
Arg	Leu	Asn	MET	His	MET	Asn	Val	Gln	Asn	Gly	Lys	Trp	Asp	Ser	Asp	Pro	Ser			
										40									50	
GGG	ACC	AAA	ACC	TGC	ATT	GAT	ACC	AAG	GAA	GGC	ATC	CTG	CAG	TAT	TGC	CAA	GAA			
Gly	Thr	Lys	Thr	Cys	Ile	Asp	Thr	Lys	Glu	Gly	Ile	Leu	Gln	Tyr	Cys	Gln	Glu			
										60									70	
GTC	TAC	CCT	GAA	CTG	CAG	ATC	ACC	AAT	GTG	GTA	GAA	GCC	AAC	CAA	CCA	GTG	ACC			
Val	Tyr	Pro	Glu	Leu	Gln	Ile	Thr	Asn	Val	Val	Glu	Ala	Asn	Gln	Pro	Val	Thr			
										80									90	
ATC	CAG	AAC	TGG	TGC	AAG	CGG	GGC	CGC	AAG	CAG	TGC	AAG	ACC	CAT	CCC	CAC	TTT			
Ile	Gln	Asn	Trp	Cys	Lys	Arg	Gly	Arg	Lys	Gln	Cys	Eys	Thr	His	Pro	His	Phe			
										100									110	
GTG	ATT	CCC	TAC	CGC	TGC	TTA	GTT	GGT	GAG	TTT	GTA	AGT	GAT	GCC	CTT	CTC	GTT			
Val	Ile	Pro	Tyr	Arg	Cys	Leu	Val	Gly	Glu	Phe	Val	Ser	Asp	Ala	Leu	Leu	Val			
										120										
CCT	GAC	AAG	TGC	AAA	TTC	TTA	CAC	CAG	GAG	AGG	ATG	GAT	GTT	TGC	GAA	ACT	CAT			
Pro	Asp	Lys	Cys	Lys	Phe	Leu	His	Gln	Glu	Arg	MET	Asp	Val	Cys	Glu	Thr	His			
										130									140	
CTT	CAC	TGG	CAC	ACC	GTC	GCC	AAA	GAG	ACA	TGC	AGT	GAG	AAG	AGT	ACC	AAC	TTG			
Leu	His	Trp	His	Thr	Val	Ala	Lys	Glu	Thr	Cys	Ser	Glu	Lys	Ser	Thr	Asn	Leu			
										150									160	
CAT	GAC	TAC	GGC	ATG	TTG	CTG	CCC	TGC	GGA	ATT	GAC	AAG	TTC	CGA	GGG	GTA	GAG			
His	Asp	Tyr	Gly	MET	Leu	Leu	Pro	Cys	Gly	Ile	Asp	Lys	Phe	Arg	Gly	Val	Glu			
										170									180	
TTT	GTG	TGT	TGC	CCA	CTG	GCT	GAA	GAA	AGT	GAC	AAT	GTG	GAT	TCT	GCT	GAT	GCG			
Phe	Val	Cys	Cys	Pro	Leu	Ala	Glu	Glu	Ser	Asp	Asn	Val	Asp	Ser	Ala	Asp	Ala			
										190									200	

FIG. 1-1

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GAG	GAG	GAT	GAC	TCG	GAT	GTC	TGG	TGG	GGC	GGA	GCA	GAC	ACA	GAC	TAT	GCA	GAT
Glu	Glu	Asp	Asp	Ser	Asp	Val	Trp	Trp	Gly	Gly	Ala	Asp	Thr	Asp	Tyr	Ala	Asp
210																	
GGG	AGT	GAA	GAC	AAA	GTA	GTA	GAA	GTA	GCA	GAG	GAG	GAA	GAA	GTG	GCT	GAG	GTG
Gly	Ser	Glu	Asp	Lys	Val	Val	Glu	Val	Ala	Glu	Glu	Glu	Glu	Val	Ala	Glu	Val
220																	
GAA	GAA	GAA	GAA	GCC	GAT	GAT	GAC	GAG	GAC	GAT	GAG	GAT	GGT	GAT	GAG	GTA	GAG
Glu	Glu	Glu	Glu	Ala	Asp	Asp	Asp	Glu	Asp	Asp	Glu	Asp	Gly	Asp	Glu	Val	Glu
240																	
GAA	GAG	GCT	GAG	GAA	CCC	TAC	GAA	GAA	GCC	ACA	GAG	AGA	ACC	ACC	AGC	ATT	GCC
Glu	Glu	Ala	Glu	Glu	Pro	Tyr	Glu	Glu	Ala	Thr	Glu	Arg	Thr	Thr	Ser	Ile	Ala
260																	
ACC	ACC	ACC	ACC	ACC	ACC	ACA	GAG	TCT	GTG	GAA	GAG	GTG	GTT	CGA	GAG	GTG	TGC
Thr	Thr	Thr	Thr	Thr	Thr	Thr	Glu	Ser	Val	Glu	Glu	Val	Val	Arg	Glu	Val	Cys
280																	
TCT	GAA	CAA	GCC	GAG	ACG	GGG	CCG	TGC	CGA	GCA	ATG	ATC	TCC	CGC	TGG	TAC	TTT
Ser	Glu	Gln	Ala	Glu	Thr	Gly	Pro	Cys	Arg	Ala	MET	Ile	Ser	Arg	Trp	Tyr	Phe
300																	
GAT	GTG	ACT	GAA	GGG	AAG	TGT	GCC	CCA	TTC	TTT	TAC	GGC	GGA	TGT	GGC	GGC	AAC
Asp	Val	Thr	Glu	Gly	Lys	Cys	Ala	Pro	Phe	Phe	Tyr	Gly	Gly	Cys	Gly	Gly	Asn
310																	
CGG	AAC	AAC	TTT	GAC	ACA	GAA	GAG	TAC	TGC	ATG	GCC	GTG	TGT	GGC	AGC	GCC	ATT
Arg	Asn	Asn	Phe	Asp	Thr	Glu	Glu	Tyr	Cys	MET	Ala	Val	Cys	Gly	Ser	Ala	Ile
330																	
CCT	ACA	ACA	GCA	GCC	AGT	ACC	CCT	GAT	GCC	GTT	GAC	AAG	TAT	CTC	GAG	ACA	CCT
Pro	Thr	Thr	Ala	Ala	Ser	Thr	Pro	Asp	Ala	Val	Asp	Lys	Tyr	Leu	Glu	Thr	Pro
350																	
GGG	GAT	GAG	AAT	GAA	CAT	GCC	CAT	TTC	CAG	AAA	GCC	AAA	GAG	AGG	CTT	GAG	GCC
Gly	Asp	Glu	Asn	Glu	His	Ala	His	Phe	Gln	Lys	Ala	Lys	Glu	Arg	Leu	Glu	Ala
370																	
AAG	CAC	CGA	GAG	AGA	ATG	TCC	CAG	GTC	ATG	AGA	GAA	TGG	GAA	GAG	GCA	GAA	CGT
Lys	His	Arg	Glu	Arg	MET	Ser	Gln	Val	MET	Arg	Glu	Trp	Glu	Glu	Ala	Glu	Arg
390																	
CAA	GCA	AAG	AAC	TTG	CCT	AAA	GCT	GAT	AAG	AAG	GCA	GTT	ATC	CAG	CAT	TTC	CAG
Gln	Ala	Lys	Asn	Leu	Pro	Lys	Ala	Asp	Lys	Lys	Ala	Val	Ile	Gln	His	Phe	Gln
400																	
GAG	AAA	GTG	GAA	TCT	TTG	GAA	CAG	GAA	GCA	GCC	AAC	GAG	AGA	CAG	CAG	CTG	GTG
Glu	Lys	Val	Glu	Ser	Leu	Glu	Gln	Glu	Ala	Ala	Asn	Glu	Arg	Gln	Gln	Leu	Val
420																	
430																	

FIG. 1-2



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GAG ACA CAC ATG GCC AGA GTG GAA GCC ATG CTC AAT GAC CGC CGC CGC CTG GCC  
 Glu Thr His MET Ala Arg Val Glu Ala MET Leu Asn Asp Arg Arg Arg Leu Ala  
 440 450

CTG GAG AAC TAC ATC ACC GCT CTG CAG GCT GTT CCT CCT CGG CCT CGT CAC GTG  
 Leu Glu Asn Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val  
 460 470

TTC AAT ATG CTA AAG AAG TAT GTC CGC GCA GAA CAG AAG GAC AGA CAG CAC ACC  
 Phe Asn MET Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His Thr  
 480

CTA AAG CAT TTC GAG CAT GTG CGC ATG GTG GAT CCC AAG AAA GCC GCT CAG ATC  
 Leu Lys His Phe Glu His Val Arg MET Val Asp Pro Lys Lys Ala Ala Gln Ile  
 490 500

CGG TCC CAG GTT ATG ACA CAC CTC CGT GTG ATT TAT GAG CGC ATG AAT CAG TCT  
 Arg Ser Gln Val MET Thr His Leu Arg Val Ile Tyr Glu Arg MET Asn Gln Ser  
 510 520

CTC TCC CTG CTC TAC AAC GTG CCT GCA GTG GCC GAG GAG ATT CAG GAT GAA GTT  
 Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala Glu Glu Ile Gln Asp Glu Val  
 530 540

GAT GAG CTG CTT CAG AAA GAG CAA AAC TAT TCA GAT GAC GTC TTG GCC AAC ATG  
 Asp Glu Leu Leu Gln Lys Glu Gln Asn Tyr Ser Asp Asp Val Leu Ala Asn MET  
 550 560

ATT AGT GAA CCA AGG ATC AGT TAC GGA AAC GAT GCT CTC ATG CCA TCT TTG ACC  
 Ile Ser Glu Pro Arg Ile Ser Tyr Gly Asn Asp Ala Leu MET Pro Ser Leu Thr  
 570

GAA ACG AAA ACC ACC GTG GAG CTC CTT CCC GTG AAT GGA GAG TTC AGC CTG GAC  
 Glu Thr Lys Thr Thr Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp  
 580 590

GAT CTC CAG CCG TGG CAT TCT TTT GGG GCT GAC TCT GTG CCA GCC AAC ACA GAA  
 Asp Leu Gln Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu  
 600 610

AAC GAA GTT GAG CCT GTT GAT GCC CGC CCT GCT GCC GAC CGA GGA CTG ACC ACT  
 Asn Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr Thr  
 620 630

CGA CCA GGT TCT GGG TTG ACA AAT ATC AAG ACG GAG GAG ATC TCT GAA GTG AAG  
 Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys  
 640 650

ATG GAT GCA GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT CAT CAT CAA AAA TTG  
 MET Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu  
 660

FIG. 1-3

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GTG	TTC	TTT	GCA	GAA	GAT	GTG	GGT	TCA	AAC	AAA	GGT	GCA	ATC	ATT	GGA	CTC	ATG
Val	Phe	Phe	Ala	Glu	Asp	Val	Gly	Ser	Asn	Lys	Gly	Ala	Ile	Ile	Gly	Leu	MET
670										680							
GTG	GGC	GGT	GTT	GTC	ATA	GCG	ACA	GTG	ATC	GTC	ATC	ACC	TTG	GTG	ATG	CTG	AAG
Val	Gly	Gly	Val	Val	Ile	Ala	Thr	Val	Ile	Val	Ile	Thr	Leu	Val	MET	Leu	Lys
		690										700					
AAG	AAA	CAG	TAC	ACA	TCC	ATT	CAT	CAT	GGT	GTG	GTG	GAG	GTT	GAC	GCC	GCT	GTC
Lys	Lys	Gln	Tyr	Thr	Ser	Ile	His	His	Gly	Val	Val	Glu	Val	Asp	Ala	Ala	Val
				710										720			
ACC	CCA	GAG	GAG	CGC	CAC	CTG	TCC	AAG	ATG	CAG	CAG	AAC	GGC	TAC	GAA	AAT	CCA
Thr	Pro	Glu	Glu	Arg	His	Leu	Ser	Lys	MET	Gln	Gln	Asn	Gly	Tyr	Glu	Asn	Pro
						730										740	
ACC	TAC	AAG	TTC	TTT	GAG	CAG	ATG	CAG	AAC	TAG							
Thr	Tyr	Lys	Phe	Phe	Glu	Gln	MET	Gln	Asn								
										750							

FIG. 1-4

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TTT	TTG	TTC	AAA	ATA	GGT	AGT	AAT	27	TGA	AGT	TTT	AAA	TAT	AGG	GTA	TCA	TTT	54	TTC
Phe	Leu	Phe	Lys	Ile	Gly	Ser	Asn	.	Ser	Phe	Lys	Tyr	Arg	Val	Ser	Phe	Phe		
TTT	AAG	AGT	CAT	TTA	TCA	ATT	TTC	81	TTC	TAA	CTT	CAG	GCC	TAG	AAA	GAA	GTT	108	TTG
Phe	Lys	Ser	His	Leu	Ser	Ile	Phe	Phe	.	Leu	Gln	Ala	.	Lys	Glu	Val	Leu		
GGT	AGG	CTT	TGT	CTT	ACA	GTG	TTA	135	TTA	TTT	ATG	AGT	AAA	ACT	AAT	TGG	TTG	162	TCC
Gly	Arg	Leu	Cys	Leu	Thr	Val	Leu	Leu	Phe	MET	Ser	Lys	Thr	Asn	Trp	Leu	Ser		
TGC	ATA	CTT	TAA	TTA	TGA	TGT	AAT	189	ACA	GGT	TCT	GGG	TTG	ACA	AAT	ATC	AAG	216	ACG
Cys	Ile	Leu	.	Leu	.	Cys	Asn	Thr	Gly	Ser	Gly	Leu	Thr	Asn	Ile	Lys	Thr		
GAG	GAG	ATC	TCT	GAA	GTG	AAG	ATG	243	GAT	GCA	GAA	TTC	CGA	CAT	GAC	TCA	GGA	270	TAT
Glu	Glu	Ile	Ser	Glu	Val	Lys	MET	Asp	Ala	Glu	Phe	Arg	His	Asp	Ser	Gly	Tyr		
GAA	GTT	CAT	CAT	CAA	AAA	TTG	GTA	297	CGT	AAA	ATA	ATT	TAC	CTC	TTT	CCA	CTA	324	CTG
Glu	Val	His	His	Gln	Lys	Leu	Val	Arg	Lys	Ile	Ile	Tyr	Leu	Phe	Pro	Leu	Leu		
TTT	GTC	TTG	CCA	AAT	GAC	CTA	TTA	351	ACT	CTG	GTT	CAT	CCT	GTG	CTA	GAA	ATC	378	AAA
Phe	Val	Leu	Pro	Asn	Asp	Leu	Leu	Thr	Leu	Val	His	Pro	Val	Leu	Glu	Ile	Lys		
TTA	AGG	AAA	AGA	TAA	AAA	TAC	AAT	405	GCT	TGC	CTA	TAG	GAT	TAC	CAT	GAA	AAC	432	ATG
Leu	Arg	Lys	Arg	.	Lys	Tyr	Asn	Ala	Cys	Leu	.	Asp	Tyr	His	Glu	Asn	MET		
AAG	AAA	ATA	AAT	AGG	CTA	GGC	TGA	459	GCG	CAG	TGG	CTC	AAG	CCT	GTA	ATC	CCA	486	GCA
Lys	Lys	Ile	Asn	Arg	Leu	Gly	.	Ala	Gln	Trp	Leu	Lys	Pro	Val	Ile	Pro	Ala		

FIG. 2

FIG. 3-1

10 20 30 40 50 60 70  
GAATTCCT GGGAGCCAA GGAATTGGGA ATGTGTAGCC CAAGTAAGAC AGAACCAGC AGGAACATGC

80 90 100 110 120 130 140  
CTCTCCTTAG GGTGCTGATA CCTGTTCAAG GTTTTAAATGT GGAAGGGAGG ATTAGGCTTG CTCTGTGTTG

150 160 170 180 190 200 210  
AATCAGGCTC AAAGGATGGA AGTTACAGGG AAGCTGATTC TGGCTTCATG TAAAAAAGG ACAGTTTGGG

220 230 240 250 260 270 280  
CAGGCNAATC TATCAAAAAA TGGAGGGGAA TTGATACATT CCTCTATGTT CAAACAGGAA CTGACAATCT

290 300 310 320 330 340 350  
GCCCCCTGGT GGAACACCGG TAGAGAGAT GACTTCAAA GCCCTTTCA TCCTAAATTT CTGATGTTG

360 370 380 390 400 410 420  
ATAATTAAAT GTTATAGCAT GGACACTGAC ATTTACATTT TTTACTTATG TTTTGGTTT TTAATGACT

430 440 450 460 470 480 490  
CTGCATTTTG TTTTAAGCTT CAAATTATTA TTTGAATAAT GAAATTCATC AGAACAATTA GTGTTAAGAA

500 510 520 530 540 550 560  
TCATATAGCA ATTTATAGAA AAGGAAGAGT TCGTAGGTTA TAAATTCTGT TAGTTGCTAA GAAGCATTTT

570 580 590 600 610 620 630  
TAAATTATG TACTATAGCT CTTTATTCAG CAGACGAACC AATTACAATC TGTGTAAC TA GAACACTTGA

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## FIG. 3-2

640 CTAAATAT ATAATTTTA CAACGCTTCA CTGCATAGAT ACATGAACAT AATTATTG TAATTGGAAC 700  
 710 AAAGCCCCAA AGTAGCAGTT TTGTTCTACC AGGTAATTA TGCTCATTTT TAAAGCCCTT TATTATTATT 770  
 780 TCTGAAGTAA TGAGTGCACA TGGAAAAGA CACATAATAG GCTAAACAAT AAGCCCGTAA GCCAAGCCAA 840  
 850 CATATTCCAG GAACAAATCC TTGCCAACCT CTCAACCCAGG ATTAACTTC TGCTTTTCCC CCATTITCAA 910  
 920 AAATTATAGC ATGTATTAA AGGCAGCAGA AGCCTTACTT TCAGGTTTCC CTTACCCCTT CATTTCTTTT 980  
 990 TGTTCAAAAT AGGTAGTAAT TGAAGTTTA AATATAGGGT ATCATTTTTC TTTAAGAGTC ATTTATCAAT 1050  
 1060 TTTCTTCTAA CTTCAGGCCT AGAAGAAGT TTTGGGTAGG CTTTGCTCTA CAGTGTATT ATTTATGAGT 1120  
 1130 AAAACTAAT GGTGTCTCTG CATACTTAA TTATGATGTA ATACAGGTTT TGGGTTGACA AATATCAAGA 1190  
 1200 CGGAGGAGAT CTCTGAAGTG AAG ATG GAT GCA GAA TTC 1228  
 MET Asp Ala Glu Phe

1 2 3 4

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## FIG. 4-1

GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT CAT CAT CAA AAA TTG GTG TTC TTT  
 Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu Val Phe Phe  
 3 10 20  
 GCA GAA GAT GTG GGT TCA AAC AAA GGT GCA ATC ATT GGA CTC ATG GTG GGC GGT  
 Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu MET Val Gly Gly  
 30  
 GTT GTC ATA GCG ACA GTG ATC GTC ATC ACC TTG GTG ATG CTG AAG AAG AAA CAG  
 Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val MET Leu Lys Lys Gln  
 40 50  
 TAC ACA TCC ATT CAT GGT CAT GGT GTG GTG GAG GTT GAC GCC GCT GTC ACC CCA GAG  
 Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu  
 60 70  
 GAG CGC CAC CTG TCC AAG ATG CAG CAG AAC GGC TAC GAA AAT CCA ACC TAC AAG  
 Glu Arg His Leu Ser Lys MET Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys  
 80 90  
 TTC TTT GAG CAG ATG CAG AAC TAG ACCCCCGCCA CAGCAGCCTC TGAAGTTGGA CAGCAAAACC  
 Phe Phe Glu Gln MET Gln Asn  
 344 354 364 374 384 394 404  
 ATTGCTTCAC TACCCATCGG TGTCCATTTA TAGAATAATG TGGGAAGAAA CAAACCCGTT TTATGATTTA  
 99  
 CTCATTATCG CCTTTTGACA GCTGTGCTGT AACACAAGTA AATGCCCTGAA CTGGAATTAA TCCACACATC  
 414 424 434 444 454 464 474  
 AGTAAAGTAT TCTATCTCTC TTTACATTTT GGTCTCTATA CTACATTATT AATGGGTTTT GTGTACTGTA  
 484 494 504 514 524 534 544

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## FIG. 4-2

554 564 574 584 594 604 614  
 AAGAAATTAG CTGTATCAAA CTAGTGCATG AATAGATTCT CTCCTGATTA TTTATCACAT AGCCCCCTTAG  
  
 624 634 644 654 664 674 684  
 CCAGTTGTAT ATTATTCTTG TGGTTTGTGA CCCAATTAAAG TCCTACTTTA CATATGCTTT AAGAATCGAT  
  
 694 704 714 724 734 744 754  
 GGGGGATGCT TCATGTGAAC GTGGGAGTTC AGCTGCTTCT CTTGCCCTAAG TATTCCTTTC CTGATCACTA  
  
 764 774 784 794 804 814 824  
 TGCATTTTAA AGTTAAACAT TTTTAAGTAT TTCAGATGCT TTAGAGAGAT TTTT'TTCC'A TGACIGCAJT  
  
 834 844 854 864 874 884 894  
 TTA CTGTACA GATTGCTGCT TCTGCTATAT TTGTGATATA GGAATTAAGA GGATACACAC GTTTGTTTCT  
  
 904 914 924 934 944 954 964  
 TCGTGCCCTGT TTTATGTGCA CACATTAGGC ATTGAGACTT CAAGCTTTTC TTTT'TTGTG CACGTATCTT  
  
 974 984 994 1004 1014 1024 1034  
 TGGGTCTTG ATNAGAAA GAATCCCTGT TCATTGTAAG CACTTTTACG GGGCGGGTGG GGAGGGGTGC  
  
 1044 1054  
 TCTGCTGGTC TTCAATTACC AAGAATTC

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ATG GAT GCA GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT CAT CAT  
Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His  
0 10

CAA AAA TTG GTG TTC TTT GCA GAA GAT GTG GGT TCA AAC AAA  
Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys  
20

GGT GCA ATC ATT GGA CTC ATG GTG GGC GGT GTT GTC ATA GCG  
Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala  
30 40

ACA GTG ATC GTC ATC ACC TTG GTG ATG CTG AAC AAG AAA CAG  
Thr Val Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln  
50

TAC ACA TCC ATT CAT CAT GGT GTG GTG GAG GTT GAC GCC GCT  
Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala Ala  
60 70

GTC ACC CCA GAG GAG CGC CAC CTG TCC AAG ATG CAG CAG AAC  
Val Thr Pro Glu Glu Arg His Leu Ser Lys Met Gln Gln Asn  
80

GGC TAC GAA AAT CCA ACC TAC AAG TTC TTT GAG CAG ATG CAG  
Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met Gln  
90

AAC  
Asn

FIG. 5



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## FIG. 6

GAA TTC GGA CAT GAT TCA GGA TTT GAA GTC CGC CAT CAA AAA CTG GTG TTC TTT	27	54
Glu Phe Gly His Asp Ser Gly Phe Glu Val Arg His Gln Lys Leu Val Phe Phe		
3	10	20
GCT GAA GAT GTG GGT TCG AAC AAA GGC GCC ATC ATC GGA CTC ATG GTG GGC GGC	81	108
Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu MET Val Gly Gly		
	30	
GTT GTC ATA GCA ACC GTG	135	
Val Val Ile Ala Thr Val		
40		



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## FIG. 7-2

## Amino Acid Comparison

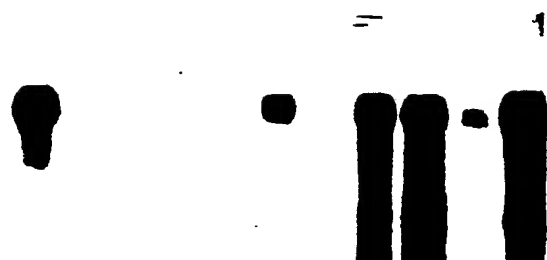
W3	Glu	Phe	Arg	His	Asp	Ser	Gly	Tyr	Glu	Val	His	His	Gln	Lys	Leu	Val	Phe	Phe
			X				X											
W9	Glu	Phe	Gly	His	Asp	Ser	Gly	Phe	Glu	Val	Arg	His	Gln	Lys	Leu	Val	Phe	Phe

W3	Ala	Glu	Asp	Val	Gly	Ser	Asn	Lys	Gly	Ala	Ile	Ile	Gly	Leu	MET	Val	Gly	Gly
W9	Ala	Glu	Asp	Val	Gly	Ser	Asn	Lys	Gly	Ala	Ile	Ile	Gly	Leu	MET	Val	Gly	Gly

W3	Val	Val	Ile	Ala	Thr	Val
W9	Val	Val	Ile	Ala	Thr	Val

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1 2 3 4 5 6 7 8 9 10 11



-28s FIG. 8A

Junction

-18s



-28s FIG. 8B

Insert

-18s



-28s FIG. 8C

Actin

-18s

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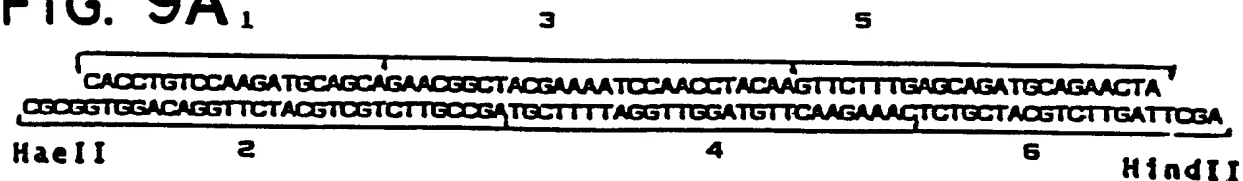
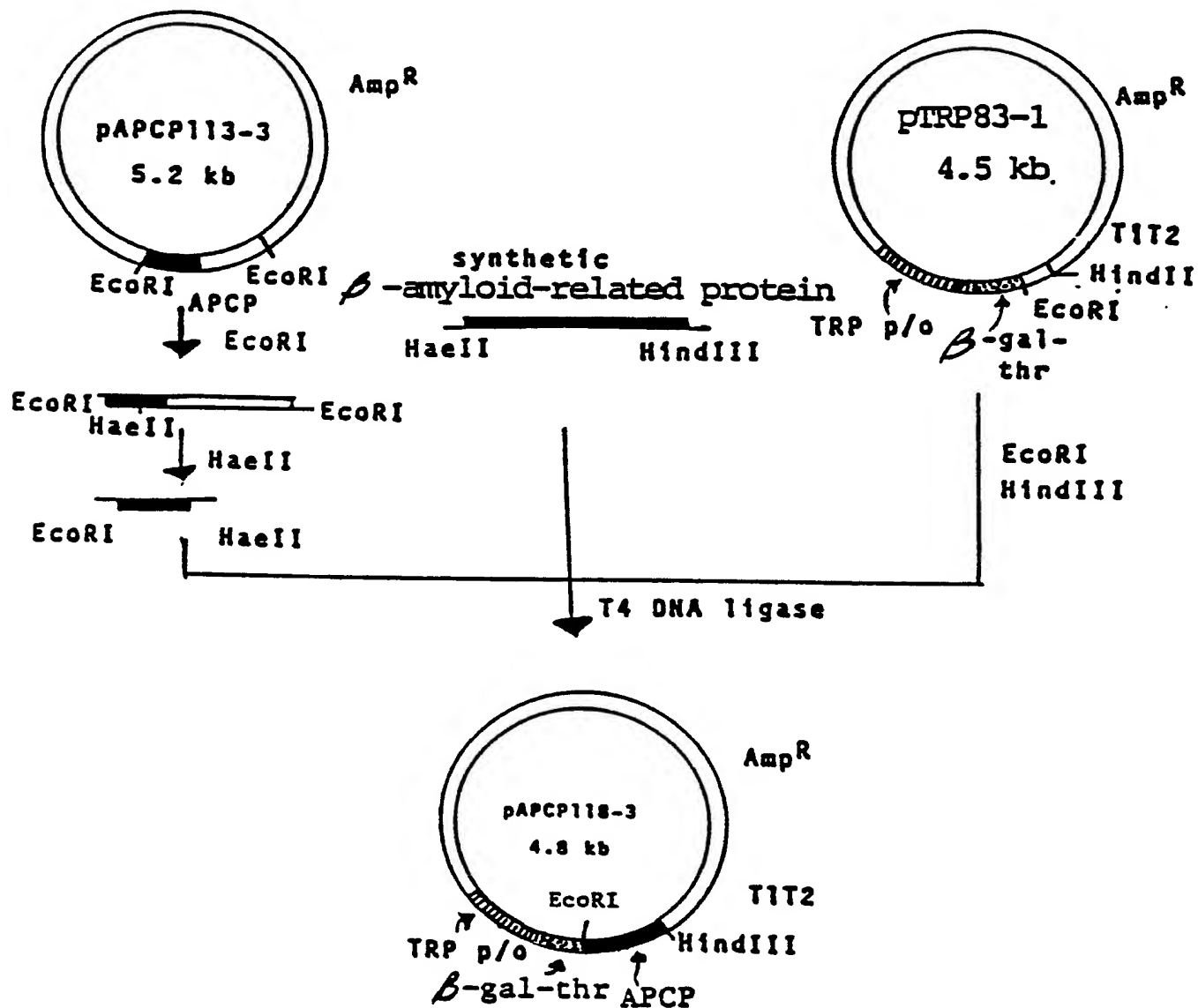
FIG. 9A<sub>1</sub>

FIG. 9B



## FIG. 9C

NH<sub>2</sub>-Met-Thr-Ile-Thr-Leu-Thr-Thr-Thr-Thr-Thr- (beta-gal-thr leader).

655

Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-  
 Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-  
 Ile-Ala-Thr-Val-Ile-Val-Ile-Thr-Leu-Val-Met-Leu-Lys-Lys-Lys-Gln-Tyr-Thr-Ser-  
 Ile-His-His-Gly-Val-Val-Glu-Val-Asp-Ala-Ala-Val-Thr-Pro-Glu-Glu-Arg-His-Leu-  
 Ser-Lys-Met-Gln-Gln-Asn-Gly-Tyr-Glu-Asn-Pro-Thr-Tyr-Lys-Phe-Phe-Glu-Gln-Met-

751

Gln-Asn-COOH (8-amyloid-related polypeptide)

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## FIG. 9D

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GluPheAsnGlyGluValCysSerGluGlnAlaGluThrGlyProCysArgAla2Het1IleSerArgIrpIyrPheAspVal  
AATTCAACGGGAGGTGCTCTGAACAAGCTGAGACTGGCCCGTGGCGTGCATGATCTCCCGCTGGTACTTTGATGTG  
 GTTGGCGCTCCACACGAGACTTGTTCGACTCTGACCGGGCACGGCAGTTAETAGAGGGCGACCAIGAAACTACAC  
 EcoRI

ThrGluGlyLysCysAlaProPhePheTyrGlyGlyCysGlyGlyAsnArgAsnAsnPheAspThrGluGluTyrCysMet  
ACTGAAGGTAAAGTGGCTCTCATTTCTTTACGGCGGTTGCGGGCAACCGTAACAACCTTTGACACTGAAGAGTACTGCAIG  
TGACTTCCATTACCGCGAGGTAAGAAATGCCGCCAACCGCGCGTTGGCAITGTIGAAACIGTACTTCTCAIGACGTAC

345

AlaValCysGlySerAlaIleTER  
GCAGTGTGGCGAGCGCTATTTAAGGATCCA'  
CGTCACACGCCGTCGCGATAAATTCCTAGGTTCGA,  
 BamHIIHindIII

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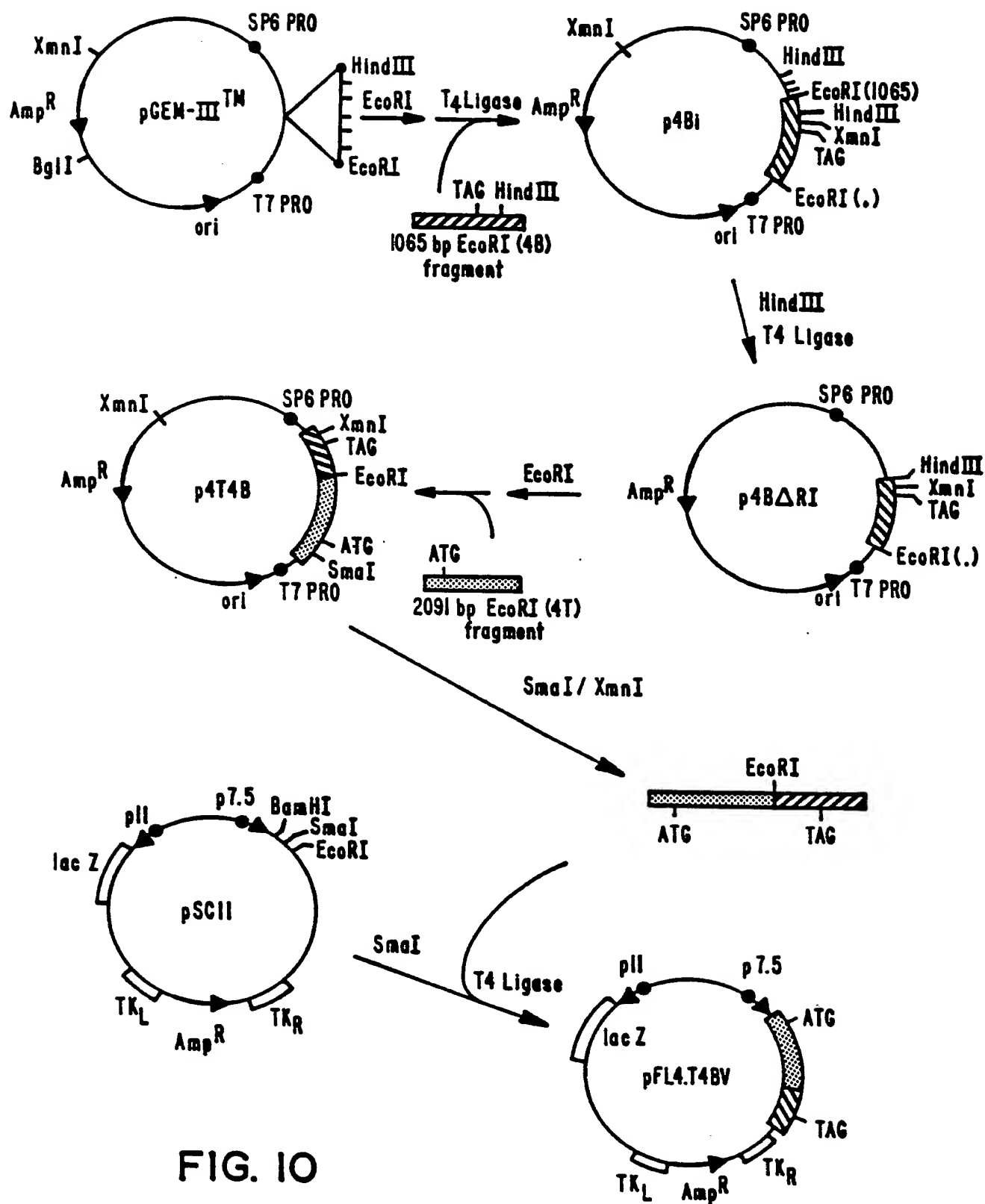


FIG. 10



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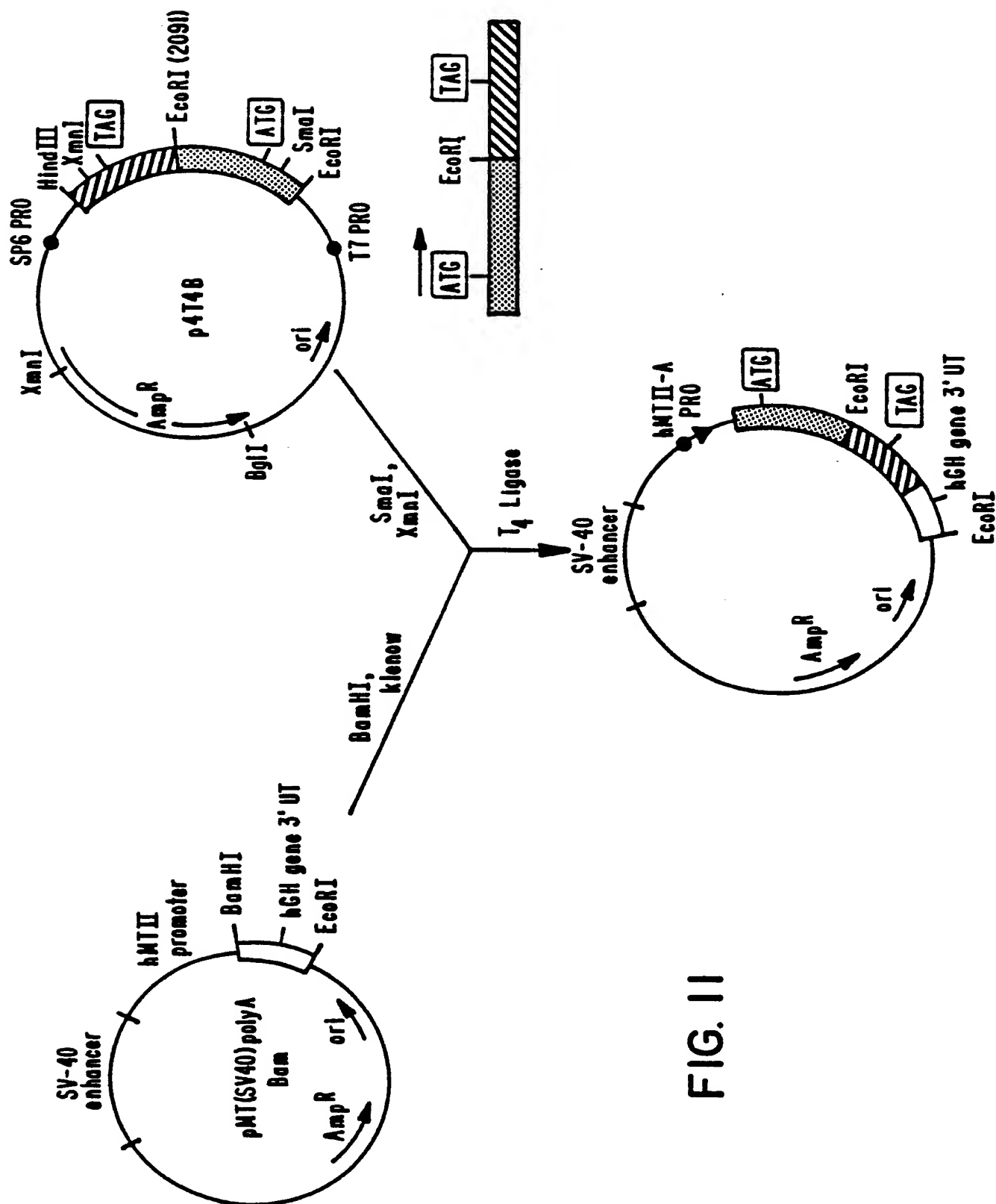


FIG. II

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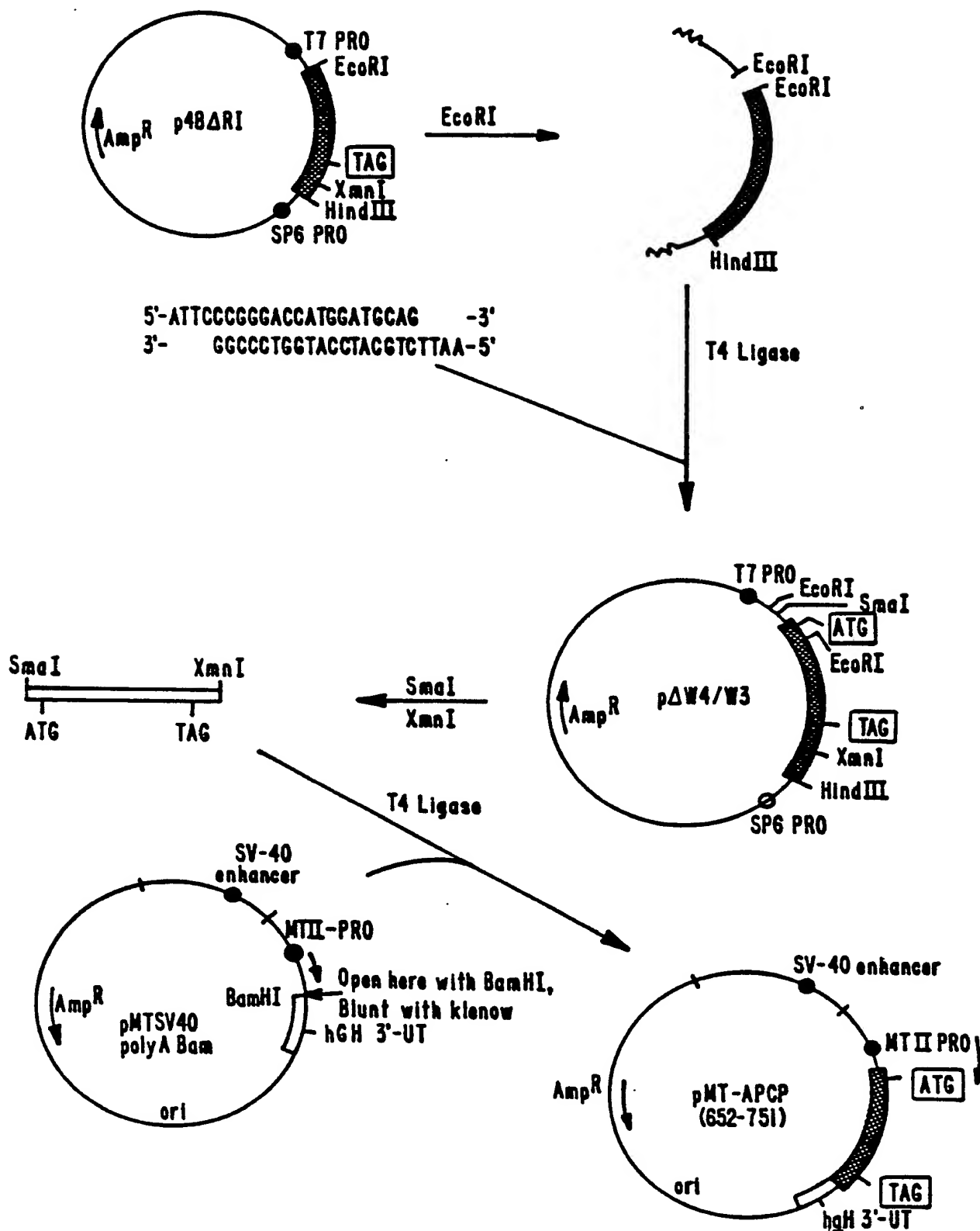


FIG. 12

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## FIG. 13-1

TIHUBI : Inter-alpha-trypsin inhibitor (BPI type)  
50.0% identity in 52 aa overlap

1" AVLPQEEGSGGQLVTEVTKKEDSCQLGYSAGPCMGHTSRFYNGTSMACETFYGGGCM

INSERT

1'

EVCSEQAETGPCRAMISRHWYFDVTEGKCAPPFYGGCGGNRN

TIHUBI

61"

GNGNPFVTEKECLQTCRTVAACNLPVIRGPCRAFIQLHAFDAVKGKCVLPFYGGCQGN

42' NFDTEYCHAVCGSAI

:: :: :: :: ::

121" KFYSEKREYCGVPGDEDELL

TIBOBI : Inter-alpha-trypsin inhibitor (BPI type)  
40.1% identity in 54 aa overlap

INSERT

1'

EV

TIBOBI

1"

KADSCQLDYSQGPCGLFKRYFYNGTSMACETFLYGGCHGNLNNFLSQKECLQTCRTVEA

3'

CSEQAETGPCRAMISRHWYFDVTEGKCAPPFYGGCGGNRNFDTEYCHAVCGSAI

61"

CNLPVQGPCRAFIQLHAFDAVKGKCVRFSGGCKGNKGFYSQKECKEYCGIPGEADER

121" LL

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## FIG. 13-2

TIBO : Basic protease inhibitor precursor - Bovine  
47.4% identity in 57 aa overlap

```

INSERT 1'      EVCSEQAETGPCRAMISRWFYFDVTEGKCAPFFYGGCGGNRRNFD
TIBO      1" PSLFNRDPPIPAAQRPDFCLEPPYTGPCAKARIIRYFYNAKAGLCQTFVYGGCRAKRNFK

```

```

45' TEEYCHAVCGSAI
..: : : : :
61" SAEDCHRTCGGAIGFWGKTGGRAEGEGKG

```

TIBOR : Serum basic protease inhibitor - Bovine  
42.9% identity in 56 aa overlap

```

INSERT 1'      EVCSEQAETGPCRAMISRWFYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSA
TIBOR      1" TERPDFCLEPPYTGPCAAHIRYFYNAKAGFCETFFVYGGCRAKSNFKSAEDCHRTCGGA

```

57' I

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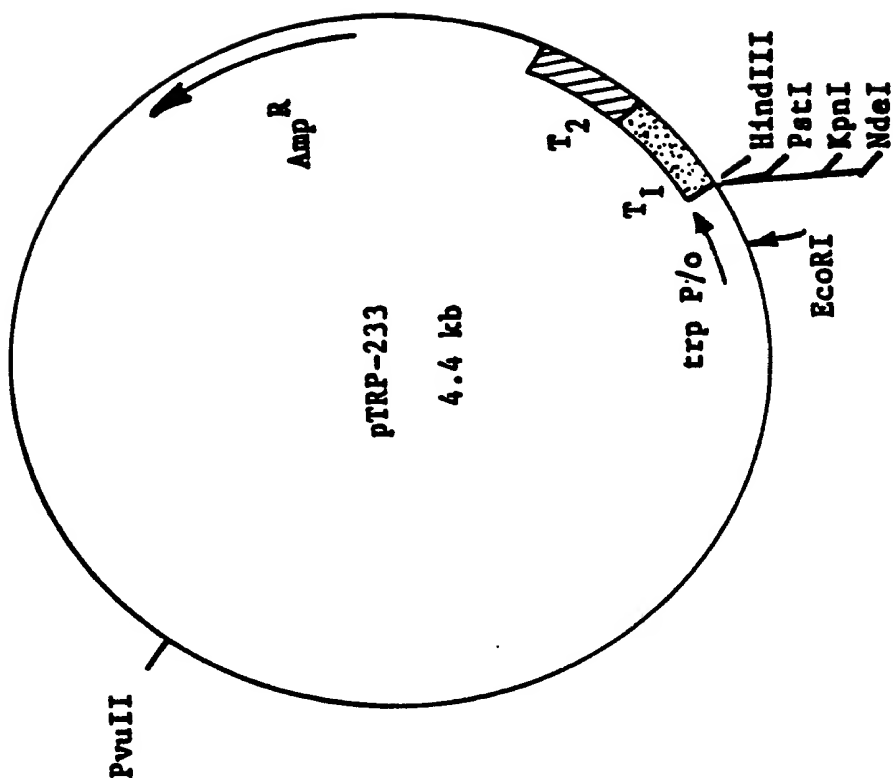
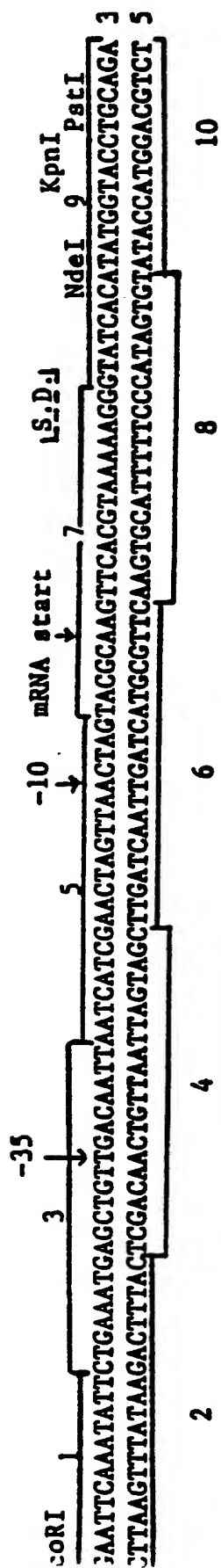


FIG. 14

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CV-1 CELLS INFECTED  
WITH A4<sub>75I</sub>-VACCINIA VIRUS  
Western Blot

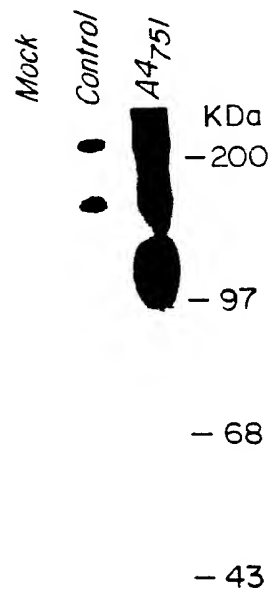


FIG. 15

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A.

NdeI

TATG AAA AAG ~~ACA~~ GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC  
 TAC TTT TTC ~~TGT~~ CGA TAG CGC TAA CGT CAC CGT GAC CGA CCA AAG CGA TGG  
 Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala Thr

10

GTA GCG CAG GCC\* GAG GTG TGC TCT GAA CAA GCT GAG ACT GGC CCG TGC CGT GCA  
 CAT CGC GTC ~~GG~~ CTC CAC ACG AGA CTT GTT CGA CTC TGA CCG GGC ACG GCA CGT  
 Val Ala Gln Ala Glu Val\* Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala

20

30

ATG ATC TCC\* CGC TGG TAC TTT GAT GTG ACT GAA GGT AAG TGC GCT CCA TTC TTT  
 TAC TAG AGG GCG ACC ATG AAA CTA CAC TGA CTT CCA TTC ACG CGA GGT AAG AAA  
 Met Ile Ser Arg Trp\* Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe

40

50

TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC\* TTT GAC ACT GAA GAG TAC TGC ATG  
 ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG TGA CTT CTC ATG ACG TAC  
 Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp\* Thr Glu Glu Tyr Cys Met

60

70

BamHI

GCA GTG TGC ~~GGC~~ AGC GCT ATT TAA GGATCCA  
 CGT CAC ACG ~~CCG~~ TCG CGA TAA ATT CCTAGGTTCTGA  
 Ala Val Cys Gly Ser Ala Ile

HindIII

B.

NdeI

TATG AAA CAA AGC ACT ATT GCA ATG GCA CTC TTA CCG TTA CTG TTT ACC CCT  
 TAC TTT GTT TCG TGA TAA CGT TAC CGT GAG AAT GGC AAT GAC AAA TEG GGA  
 Met Lys Gln Ser Thr Ile Ala Met Ala Leu Leu Pro Leu Leu Phe Thr Pro

10

GTG ACA AAA GCC\* GAG GTG TGC TCT GAA CAA GCT GAG ACT GGC CCG TGC CGT GCA  
 CAC TGT TTT CGG CTC CAC ACG AGA CTT GTT CGA CTC TGA CCG GGC ACG GCA CGT  
 Val Thr Lys Ala Glu Val\* Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala

20

30

ATG ATC TCC\* CGC TGG TAC TTT GAT GTG ACT GAA GGT AAG TGC GCT CCA TTC TTT  
 TAC TAG AGG GCG ACC ATG AAA CTA CAC TGA CTT CCA TTC ACG CGA GGT AAG AAA  
 Met Ile Ser Arg Trp\* Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe

40

50

TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC\* TTT GAC ACT GAA GAG TAC TGC ATG  
 ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG TGA CTT CTC ATG ACG TAC  
 Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp\* Thr Glu Glu Tyr Cys Met

60

70

BamHI

GCA GTG TGC GGC AGC GCT ATT TAA GGATCCA  
 CGT CAC ACG CCG TCG CGA TAA ATT CCTAGGTTCTGA  
 Ala Val Cys Gly Ser Ala Ile

HindIII

FIG. 16

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US90/03141

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC  
 IPC(5): A61K 37/64; C07K 7/10; C12N 15/12; C12P 21/02; C12Q 1/68  
 US CL.: 530/326; 514/12; 536/27; 435/69.1, 6

## II. FIELDS SEARCHED

Minimum Documentation Searched \*

Classification System

Classification Symbols

U.S. 530/326; 514/12; 536/27; 435/69.1, 6

Documentation Searched other than Minimum Documentation  
 to the Extent that such Documents are Included in the Fields Searched \*

Sequence Search (Swiss-Prot, PIR, CAS registry), Chemical Abstract Service,  
 Automated Patent Search

## III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 15 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 14
$\frac{X}{Y}$	Nature, "Protease Inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease", Volume 331, pages 528-530. Tanzi et al 11 February 1988. See figure 2.	$\frac{1-10, 24-32}{11-14, 16}$
$\frac{X}{Y}$	Nature, "Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity", Volume 331, pages 530-532 Kitaguchi et al, 11 February 1988. See figure 2.	$\frac{1-10, 24-32}{11-14, 16}$
$\frac{X}{Y}$	Nature, "A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors", Volume 331, pages 525-527 Ponte et al, 11 February 1988. See figure 1.	$\frac{1-10, 24-32}{11-14, 16}$
$\frac{A}{Y}$	Nature, "Enter a protease inhibitor", Volume 331, pages 478-479, Carrell 11 February 1988. See entire article.	$\frac{1-10, 15, 17-32}{11-14, 16}$
$\frac{A}{Y}$	US,A, 4,666,829 (Glenner et al) 19 May 1987 See entire document, abstract	$\frac{1-16, 24-32}{17-23}$

\* Special categories of cited documents: 13

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search \*

18 July 1990

International Searching Authority \*

ISA/US

Date of Mailing of this International Search Report \*

24 AUG 1990

Signature of Authorized Officer 29

For

Nina Ossana Ph.D.

*Nina Ossana*  
 INTERNATIONAL DIVISION



## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

$\frac{A}{Y}, P$	US,A, 4,912,206 (Goldgaber et al) 27 March 1990 See entire abstract, patent	$\frac{1-16, 24-32}{17-23}$
Y	US,A, 4,595,674 (Tschesche et al) 17 June 1986 See tables 1 and 2.	11-14, 16

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter<sup>1</sup> not required to be searched by this Authority, namely:

2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out<sup>1</sup>, specifically:

3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.